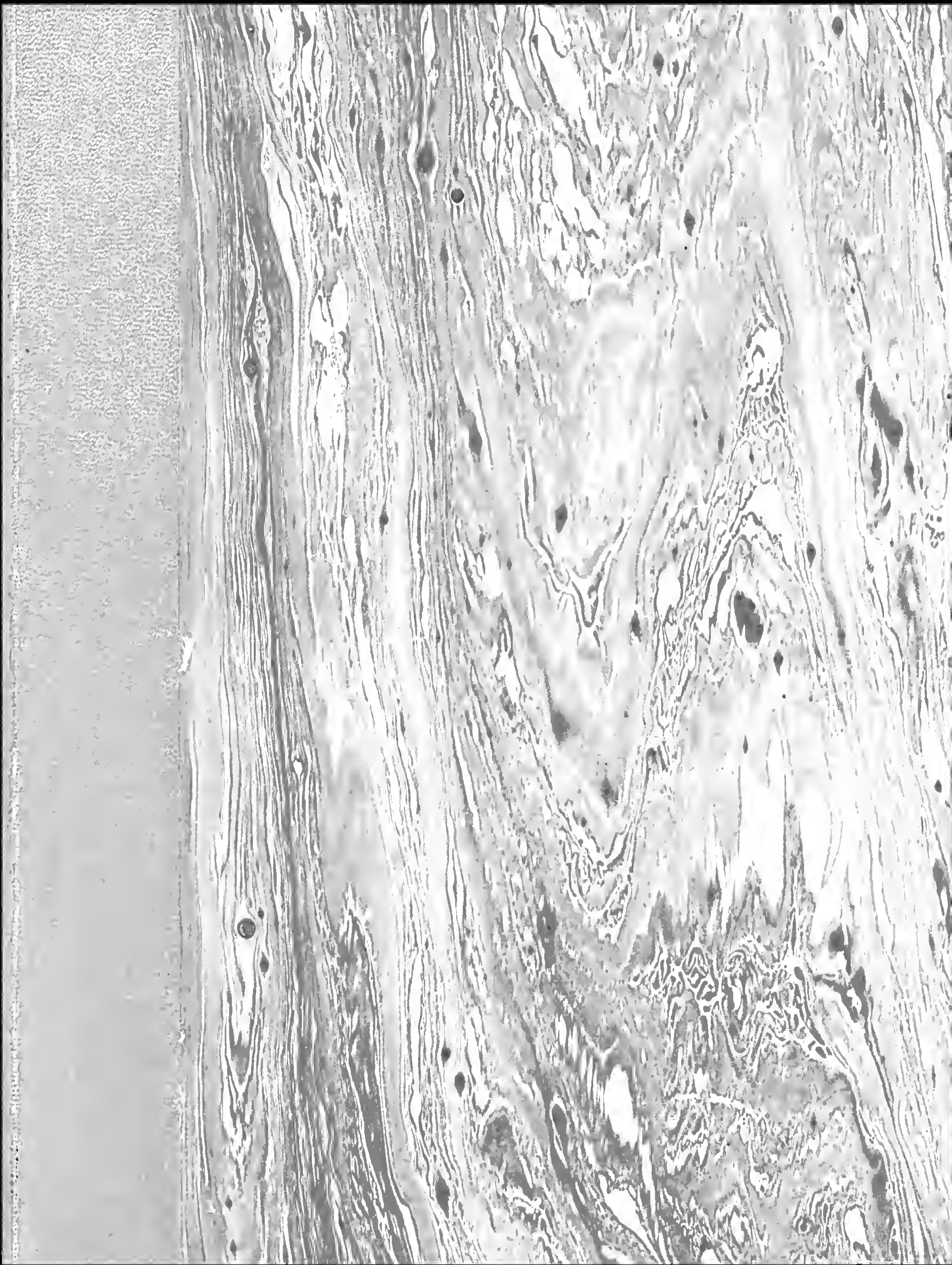

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VOLUME XXXIII

SUPLEMENTO COMEMORATIVO

SIMPÓSIO INTERNACIONAL SÔBRE VENENOS ANIMAIS

INTERNATIONAL SYMPOSIUM ON ANIMAL VENOMS

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INTERNATIONAL SYMPOSIUM ON ANIMAL VENOMS

INSTITUTO BUTANTAN

17 a 23 de julho de 1966

Sob os auspícios do GOVÊRNO DO ESTADO DE SÃO PAULO, do CONSELHO ESTADUAL DE AUXÍLIOS E SUBVENÇÕES, da SOCIEDADE DE BIOLOGIA DE SÃO PAULO, da FUNDAÇÃO DE AMPARO À PESQUISA DO ESTADO DE SÃO PAULO e do FUNDO DE PESQUISAS DO INSTITUTO BUTANTAN.

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INTERNATIONAL SYMPOSIUM ON ANIMAL VENOMS

1966

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I

ANIMAIS VENENOSOS

VENOMOUS ANIMALS

1. VENOMOUS COELENTERATES

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An important aspect of the position held by COELENTERATES as poisonous animals should be emphasized immediately. All COELENTERATES, including parasitic species, produce organelles with an elaborate structure filled with a toxic substance. It is precisely this fact that makes them specially interesting as poisonous animals since their tissues are not poisonous in themselves when ingested by other animal species including man, there being a single doubtful exception (*Physalia physalis*). They are venomous in the sense that, as a phylogenetic unit they have developed an unique and very specialized system by which the venoms they produce are injected into the tissues of the prey or any offensive body that comes into contact with the coelenterate's tissues, noticeably the epidermis of the tentacles, mouth rim and of the acontia of anemones. This very specialized system is represented by the organelles mentioned above which have a complex genesis and structure. They are typical of the COELENTERATES (*CNIDARIA*) and they are called nematocysts, cnidocysts or nettle cells.

They are ovoid or elongated capsules, filled with toxin. Before being discharged they contain the butt and the tube usually coiled spirally around the butt. Upon discharge, the hollow thread and butt are evaginated inside out and function as a hypodermic syringe, emptying the contents of the capsule into the foreign tissues which have been traversed by the tube upon explosion. Discharge is abrupt, usually explosive.

Nematocysts are usually contained in small groups of a few in an epidermal cell provided with a cnidocil that plays a part in the discharge of the nematocysts themselves. The nematocyst carrying epidermal cell is frequently erroneously called a cnidoblast, but it does not usually produce the nettle cells, it just houses them after they have been completely formed by the deeper laying nematoblasts which derive directly from interstitial undifferentiated cells. Discharge may be caused by either mechanical or chemical stimuli or both. Neither agent can be said to be the universal discharge-causing factor. Nematocysts are independent effectors (1) and their discharge is not controlled by the nervous system nor is the stimulus for discharge transmitted by the nervous net. Structure and ultrastructure of the nematocysts and of the cnidocil (2) have been described. Nematocysts are very important systematic and phylogenetic characters to be taken into account (3). The degree of intraspecific variation was shown by Ito (4) and Vannucci (5) for a hydroid and a medusa respectively, their geographic variation by Ito (6) and their importance to differentiate hydroids superficially identical belonging to different species of different families was shown by Brinckmann & Petersen (7).

Actually COELENTERATES are not the first animals in the animal kingdom who have evolved organelles specialized in defense, offense and capture of prey. The trichocysts of ciliates have certain similarities to nematocysts and the polar capsules of **CNIDOSPORIDIA** are extraordinarily similar to them. **CNIDOSPORIDIA** however, on the grounds of their morphology, life cycles and other affinities must be placed among the **SPOROZOA**. The polar capsules also have a spirally coiled thread which is evaginated upon discharge and is used to attach the spore to the inner surface of the host's gut. As far as I know, the polar capsule of the **CNIDOSPORIDIA** have no toxic action. This leaves us with COELENTERATES as the only nematocyst producers in the animal kingdom.

Summing up, the toxic effect of COELENTERATES is due to their specialized organelles which inject toxic substances in other animals, when they come in contact with them. Any animal producing nematocysts is a COELENTERATE and inversely, all COELENTERATES have nematocysts As far as I know there is a single exception to this rule which on the other hand is still open to discussion: this is the ctenophore *Euchlora rubra* which carries nematocysts apparently produced by the animal itself and are not introduced as food (8). A small diversion is pertinent at this stage. I have emphasized the fact that COELENTERATES are animals that *produce* their own nematocysts, because there actually are some gastropod molluscs, the NUDIBRANCHATA or sea-slugs that feed on hydroids or siphonophores and are unable to digest undischarged nematocysts, they rather store them away in chambers at the tip of papillae the lumen of which is in continuity with the digestive tract of the slug. The mechanism by which the nematocysts are not digested, the migration from the lumen of the gut along the diverticula and finally into the chamber, which is provided with an external pore, is yet unexplained.. It is however true that the nematocysts, stored in the apical chamber of the papilla are still functional and they are used for defense by the slugs that erect the papillae when pursued and use the nematocysts effectively. They have been aptly called CLEPTOCNIDAE since they are stolen cnidocysts.

As mentioned earlier, I ignore instances in which the tissues of COELENTERATES have been found to be poisonous when ingested. There are some large species of jelly fishes which are normally eaten by man. We may refer to Kuragê in the Far East (Japan, also China) which indicates large **SEMAEOSTOMEAE** and **RHIZOSTOMEAE** as well as the Te-Baitari of Gilbert Islands which indicates *Carybdea alata* (CARYBDEIDAE), all SCYPHOMEDUSAE. When considering the toxins injected by the nematocysts, we must draw the difference between their effect on man and on small aquatic animals. The toxins are undoubtedly active on small animals, they have a paralyzing and finally a lethal effect and on this property is based the normal feeding behaviour of COELENTERATES which are all carnivores and predators. Only a few species are lethal to man or other large animals. About 30 cases of death by stinging from jelly fishes are known with certainty, almost all of them are from the Indo-Malayan area between northern Australia and the Asian mainland (recent review by Southcott (9).

We mentioned earlier that the toxin contained in the capsule of the nematocysts is injected in the prey as if by a hypodermic syringe. The capsule is usually about 10-20 μ long or less, it is exceptionally large in SCYPHONOPHORA as for instance in *Agalma* where they may reach a length of some 200 μ

or more. Their numbers however are so great in the epidermis of the COELENTERATES, mainly in the tentacles and mouth rim or lips, that is has been estimated that a large medusa may inject some 3 cc of toxin in a single discharge. The nature of the toxin or toxins is not known with certainty. The principal difficulties involved are two: 1) — it is difficult to isolate nematocysts in sufficient quantity uncontaminated by proteins and indols from other tissues; 2) — the presence of different nematocysts in the same and in different species each with a different function and their different effect on the prey suggests that they probably contain different toxins. Lane & Dodge (10) have elaborated a method by which nematocysts may be isolated and the toxin extracted and tested separately from other tissue extracts.

The venom always has a paralyzing effect on other animals and when it is sufficiently strong to act on man specially when contact has occurred at places where the skin is finer or at mucous membranes, it causes a strong pain that feels like stinging fire. The paralyzing effect may be due to the toxin's action on the respiratory system. This is attributed to a glycerin soluble fraction called congestin, which would also cause a congestion of the gut and respiratory mucose. The alcoholic extract called thalassin causes itching, erythema and general irritation of the affected area allied to nausea, prostration and death in guinea-pigs. A fraction called hypnotoxin has also been described which is water soluble and was extracted from Siphonophores; it causes anesthesia, somnolence and death. All these toxins, whether they have been isolated in a pure state or not, are proteins, either mucoproteins with or without an indol absorbed on them, or compounds of tetramethylammonia associated to indols or to 5-hydroxytryptamine. Solutions of tetramine chemically pure when injected, cause responses similar to those of the extract of tentacles of different Coelenterates such as to reduce the tendency to autotomy of the fiddler-crab, *Uca mordax*, and to stimulate the excised moluscan heart. According to some authors the intense pain is caused by tetramine and paralysis to quarternary bases associated to it. Lane & Dodge (10) obtained similar results by injecting the extract of nematocysts freed and isolated from other tissues by a method they devised. The lethal dose of isolated toxin in mice is of 0.037 ml/kg of a solution containing 0.201% of total N.

As mentioned above, nematocysts are independent effectors uncontrolled by the nervous net, thus discharge may be highly localized, as for instance from a fragment of a tentacle or a small portion of the lips and also occurs in anesthetized animals. Peterson & Vannucci (11) while working out of the cycle of *Koellikerina fasciculata* observed that this species starves to death when kept at temperatures lower than 11°C because at such temperatures the nematocysts fail to discharge while all other functions appear to be maintained normally. It is interesting to note that nowhere, even at greater depths and in winter, the temperature in the Mediterranean Sea falls below 13.5 or 13.8°C at most. We don't know why low temperatures inhibit nematocysts discharge in this case and the matter requires further attention since preliminary observations suggest that an enzymatic mechanism may be at play.

Southcott (9) gives a detailed list of species of medical importance. Based on my own observations and experience I may add the following one for Brazilian waters:

HYDROZOA

Macrorhynchia philippina, PLUMULARIIDAE, colonial hydroid lacking a medusa free-living stage. It looks like inoffensive extremely delicate pens growing on algae and rocks below low tide mark. They produce an intense itching on human skin, irritation with erythema and the formation of little papulae. lasting several days. Southcott records the same effects from this species in the Indo-Pacific area; he calls it *Lytocarpus philippinus.*

Olindias sambaquiensis, LIMNOMEDUSAE, is called "relojinho" or little watch by local fishermen. It has a definite nettling action, the skin is reddened and later becomes vesiculated; stings may cause dizziness, muscle pain and dyspnoea. It is especially disagreable when hands soiled by the contact are taken to the eyes, a serious conjuntivitis may ensue.

The most disagreable and painful species in Brazil is *Physalia physalis,* popularly known as "Caravela" (Portuguese man-of-war).

There are records in the older literature of lethal cases in animals who were fed on *Physalia* tissues, toxicity in this case is presumably due to the stinging of the gut by the Siphonophore's nematocysts. Any contact with *Physalia* tentacles, even the slightest are extremely painful, produce erythema. papulae similar to urticaria, pain and swelling of lymphatic system, dizziness, nausea and muscle pains lasting several days. The reaction to the stinging of *Physalia* is instant and violent, it diminishes gradually with time while the effect of the stinging of Hydroids is slower, growing gradually during the first hours.

SCYPHOZOA

Chiropsalmus quadrumanus (CARYBDEIDAE) from coastal water cause an intense dermatitis, erythema, oedema and muscular pains along with swelling of lmyphatic ganglia. Areas stung by the jelly-fish develop local inflammation and ulcerations, in severe cases this is followed by fever, dizziness and cold tremors.

In comparison, *Chiropsalmus quadrigatus* a closely related species from the Indo-Pacific, may be lethal through pulmonar oedema, weakening of the pulse, respiratory difficulties, cardial blocking and death accompanied by strong pains shortly after contact with the jelly-fish.

Accidents caused by jelly-fishes are frequently accompanied by nervous and psychic phenomena such as screaming, exaltation, depression, suicidal tendencies, dyspnoea and cardiovascular disorders.

It is interesting to point out that all the dangerous species mentioned from Brazil have closely related representatives in the Indo-Pacific area belonging to the same genus or to a closely related genus. They are all mentioned by Southcott (l. c.). It appears that all the Atlantic counterparts are much milder than their Indo-Pacific relatives.

Summing up we may say that the number of species dangerous to man is small in the Atlantic Ocean, while their numbers are large in the Orient, especially in the Malayan region, in Japan and in the Philippines. Jelly-fishes are specially dangerous because they frequently occur in very dense and sometimes large patches, they are frequently transparent and rather difficult to see in the water and further their long tentacles drift about with the current, sometimes quite far away from the medusa and are quite invisible.

Among the sea-anemones or **ANTHOZOA**, although many will produce stinging sensible to men, there are no really dangerous species. We may conclude by saying that among the **HYDROZOA**, the **MILLEPORINA** and other hydroids may affect men but not as acutely as the HYDROMEDUSAE; among the **SCYPHOZOA**, the SCYPHOMEDUSAE include the species most dangerous and some may have a lethal effect. The **ANTHOZOA** are not really dangerous and the disagreable effect produced by corals is primarily due to lacerations caused by their skeleton and the breakage of small fragments which become incrusted in the victim's wound. Their action through nematocysts is only secondary.

It is known that in relation to certain species a certain degree of immunity may be developed with time, as may be observed from fishermen that are frequently exposed to certain abundant species when handling the nets soiled by the jelly-fishes. This has been shown in relation to *Cyanea* a species of the order **SEMAEOSTOMEAE** abundant in the northern seas. Probably the same happens here when there are large patches of *Olindias sambaquiensis*.

Acquired immunity may be an explanation of certain curious cases of symbiosis, difficult to explain otherwise, except perhaps by natural immunity or selection. The association between different species of **PAGURIDAE**, the hermit crabs, with certain sea-anemones has been famous for centuries, specially that between *Eupagurus bernhardus* and *Adamsia palliata*. Also the fact that young fishes, mainly cod find refuge between the hanging tentacles of large medusae, as for instance *Cyanea*, is well known and equally not fully understood. The symbiosis between certain coral fishes and sea-anemones of the family STOICHACTIDAE is less widely known but has in recent years been studied experimentally. In European aquaria, noticeably at Monaco and in American aquaria, specially at Marineland of the Pacific, one may observe fishes like *Amphiprion percula* some 5-10 cm long that live in small groups of two or three in the tentacular crown of large sea-anemones of the genus *Stoichactis*. They never swim very far from their host, they defend their territory most actively and over and over again return to the sea-anemones' tentacles, lying and rubbing against them. Neither the fish appears to be stung by the coelenterate's nematocysts nor does the sea-anemone show any attempt to feed on the fishes. A feeding behaviour would immediately be induced by any other animal behaving as that particular fish. Davenport (12) studied this behaviour experimentally. It is well ascertained that both the sea-anemone may live in symbiosis with other fishes of closely related species, and viceversa, the fish may live in symbiosis with closely related sea-anemones, either may be found isolated. *Amphiprion* which have been kept in isolation for months require several days to acclimatize to the anemone, during the process they began contact slowly and gradually with the deadly tentacles. It is believed that this is the means by which the bonds between the two species are established as well as the physiological barrier that protects the fish from the nematocysts and from being swallowed by the COELENTERATE. Davenport's experiments suggest that the mucus secreted by the fish contains an active principle that raises the threshold required for nematocyst discharge in this species of sea-anemone. This active principle is inactive at the threshold of electrically induced discharge. it acts, fast, is specific and thermolabile. The same principle is not contained in the fish's muscles.

Since a *Stoichactis* acclimated to *Amphiprion* does not exhibit the feeding reactions it shows to other fishes or to a non acclimated *Amphiprion*, it may be presumed that the active principle contained in the epi-

dermal mucus of *Amphiprion* acts on the anemone's nervous tissue a well, inhibiting its feeding reactions.

Pantin (1) studied the mechanism by which nematocysts are discharge He showed among other points that: a) nematocysts are independent effecto (recently Ross, unpublished, has expressed the view that he has evidences show that in one case at least the nervous system may interfere with nematocy discharge, see Hand (13), b) discharge of nematocysts may be stimulated b mechanical and by chemical means, among the latter specially by surface acti substances, c) solutions of usual foods make nematocysts more sensitive discharge, by mechanical means.

Thus, if the discharge threshold of nematocysts is lowered by means adequate food stuffs, inversely one must admit that the threshold for dischar must be raised by disagreable substances. If the mucus secreted by *Amphipri percula* is disagreable to *Stoichactis*, its nematocysts will not be discharge and further the sea-anemone will have no feeding reactions, similar to wh happens with *Anemonia sulcata* that does not react to and therefore does n ingest and does not feed upon fragments of flesh of the same species. As f as I can see it, this may be a tentative explanation of the behaviour of *Sto chactis* and *Amphiprion* when acclimated one to the other. Furth Parker & Van Alstyne (14) showed that extract of the epidermis of the fi *Fundulus* causes the discharge of a certain number of nematocysts of th sea-anemone *Metridium* and of *Physalia* and Davenport has shown th wounded *Amphiprion* or fishes from which even a single scale is missi are no longer immune to the stinging by *Stoichactis*. Analysing all the facts the following tentative hypothesis may be drawn: *Stoichactis'* nem tocysts normally react only feebly to the mucus of the epidermis of *Amphi prion*, the anemone does not react to this fish with its normal feeding beh viour, probably due to inhibition of the feeding behaviour by the mucus. Fu ther, Davenport has shown that fishes not acclimated to the sea-anemone on gradually develop more intimate contacts with the sea-anemone. This sugges that the fish acquire immunity gradually and that the stinging and intoxicatio which it receives at first are not very effective due to the small amount discharged nematocysts. It seems to me that experiments should be carried o along these lines that may take to the explanation of one of the most interesti and obscure cases of symbiosis yet described. Information available suggests a acquired immunity of the fish to the nematocysts and a feeding behaviour the sea-anemone inhibited by the fish's mucus.

Before closing I wish to remind the audience that the present extreme modest communication was purposely just a short reminder of the venomo aspect of beautiful COELENTERATES, in a symposium that is mainly concern with such terrific animals as serpents, spiders and scorpions.

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2. CONTRIBUCIÓN AL CONOCIMIENTO DE *THERAPHOSA LEBLONDI* (LATREILLE), 1804 (*ARANEA: THERAPHOSIDAE*)

BERTA S. GERSCHMAN DE PIKELIN y RITA D. SCHIAPELLI

Museo Argentino de Ciencias Naturales "Bernardino Rivadavia".
Buenos Aires, Argentina

En nuestra visita al Natural History Museum of New York en el año 1960, tuvimos oportunidad de ver entre el material no determinado de la colección de arañas de ese Museo, un ejemplar macho de **MYGALOMORPHAE**, que a pesar de estar partido por la mitad nos llamó la atención por su enorme talla.

Aunque en nuestros estudios sobre géneros de THERAPHOSIDAE, como *Xenestis* Simon, 1891; *Acanthoscurria* Ausserer, 1871 y *Grammostola* Simon, 1892, cuyas especies pueden tener ejemplares que llegan a los 80 mm, estábamos acostumbrados a ver arañas gigantes, el ejemplar del Museo de Nueva York nos asombró, no solo por su enorme cuerpo, sino por el grosor de sus patas, cuyos fémures anteriores tienen un diámetro ántero-posterior de más de 8 mm, casi el doble del de las otras arañas de gran tamaño.

Por gentileza del Dr. Gertsch, Curator of **ARACHNIDA**, pudimos traer al Museo Argentino de Ciencias Naturales "B. Rivadavia", en préstamo y para su estudio, varios ejemplares de MYGALOMORPHAE que necesitábamos para comparación, e incluímos esa araña gigantesca que estaba sin determinar y que no conocíamos; procedente de Venezuela, Auyan-tepui, a 1.100 m. de altura, recolectada en el año 1937.

Con las claves de Simon (1892) llegamos fácilmente al género *Theraphosa* Walck., 1805 y a su especie-tipo *Th. leblondi* (Latreille), 1804. Hicimos la determinación no exentas de emoción, porque es una especie muy rara en las colecciones, y a pesar de que está muy citada en la literatura araneológica, nada se sabe de su veneno y ecología. Este se debe a que la literatura está llena de datos contradictorios porque muy pocos aracnólogos la han visto. La mayor parte de ellos repite lo dicho por otros y muchos la han confundido con arañas de gran tamaño pertenecientes a otros géneros. Por cuya razón Simon dice (1892, p. 159): "Es la única especie conocida descripta por Latreille en *Hist. nat. Crust. Ins.*, **7**, 159, 1804. El nombre de *Mygale leblondi* o *blondii* ha sido aplicado a muchas especies de gran talla que habitan América del Sud. El tipo de Latreille, traído de la Guayana por Leblond existe todavía en el Museo" (Se refiere al Museo de Paris). "Esta rara especie parece propia de la región del Maroni, de donde yo la he recibido; es por error que ha sido citada para las Antillas, Brasil y aun para Java! (Por C. L. Koch)". Mas adelante dice Simon que el género se encuentra también en el Orinoco.

Así se explica que algunos autores la hayan visto trepando por los árboles en busca de nidos de colibríes de cuyos huevos y pichones se alimentarían; otros,

dentro de cuevas en el suelo, sin tapizar, y en cuya entrada se encuentran al anochecer al acecho de sus presas.

Para algunos son arañas tímidas que tratan de huir al ser hostigadas, no siendo peligrosas. Para otros son arañas temidas en los lugares en donde viven.

Por esa razón, si bien la tarea de determinarla fué fácil, se complicó muchísimo su estudio al consultar la bibliografía, por la confusión, no solo en su ecología y distribución, sino también en los caracteres considerados para su determinación.

Finalizado este trabajo y después de haber sido presentado nuestro "Estudio sistemático comparativo de los géneros *Theraphosa* Walck., 1805, *Lasiodora* C. L. Koch, 1851 y *Sericopelma* Ausserer, 1875" al *Simpósio Sôbre a Biota Amazônica*, Belém, Pará, Brasil, junio 6-11, 1966, tuvimos oportunidad de ver en el Museo Göldi de aquella ciudad, ejemplares macho y hembra de *Theraphosa leblondi* (Latr.), 1804, recolectados por el señor P. Waldir en Benevidez y Utinga. Por lo que dejamos establecido en contra de la opinión de Simon, que esta especie se encuentra también en el Estado de Pará, Brasil ecuatorial.

En el año 1965, el Sr. Pablo San Martín, del Uruguay, nos trajo para su estudio, varias arañas MYGALOMORPHAE procedentes de Río Caura, Departamento Bolívar, Venezuela, las que resultaron ser hembras de *Theraphosa leblondi* (Latr.), 1804, coincidentes en todos los caracteres con el ejemplar macho que teníamos en nuestro poder. Pudimos así observar las espermatecas e ilustrarlas, las que hasta ahora no se habían estudiado (carácter muy importante en la determinación de las MYGALOMORPHAE — Schiapelli & Gerschman de Pikelin, 1962).

El Sr. San Martín, que tuvo una de esas arañas viva en su laboratorio durante un año, alimentándola con insectos, nos dijo: "Fueron halladas bajo bases de hojas de palmera en la selva, en la proximidad del río. Esas bases de hojas, al desprenderse y precipitarse al suelo con la parte externa hacia arriba, constituyen un notable refugio, en forma de media caña. El lugar en donde se establece la araña está alisado. A su captura presenta una fuerte defensa y gran agresividad, la que también se observó en la araña mantenida viva en el laboratorio."

Es curioso, y lo hacemos notar aquí, que el ejemplar traído de Nueva York fué partido por la mitad con un machete por el que la recolectó. Latreille dice que el ejemplar típico de la especie que denominó *leblondi* fué muerto con la espada por el Dr. Leblond. Según parece la vista de esa araña despierta el espíritu guerrero de sus colectores.

El aporte de San Martín fué de gran valor para nuestra investigación. En las arañas MYGALOMORPHAE es muy difícil la determinación de los ejemplares hembras por falta de genitalia externa, epigino, que se encuentra en las arañas ARANEOMORPHA, habiendo sido muchas veces confundidos los distintos géneros y aun determinados como holotipos hembras de espécies nuevas, ejemplares machos jóvenes. En los machos adultos el problema no es tan difícil, porque su genitalia externa se ve fácilmente en los tarsos de los palpos y permite su identificación, lo que está facilitado por los caracteres sexuales secundarios que se encuentran presentes.

Con el material a nuestra disposición, 1 macho y 5 hembras, que pudimos comparar con los tipos macho y hembra, remitidos por el Prof. Dr. Max Vachon, del Muséum National d'Histoire Naturelle de Paris, en donde se encuentran

depositados, damos en este trabajo la diagnosis de la especie y de acuerdo a nuestro sistema, precedida por la bibliografía temática; (*B*: bibliografía; *Cat*: catálogo; *Cl*: clave; *Distr*: distribución geográfica; *L*: lista; *Morf*: morfología; *Ref*: referencia; *Syn*: sinonimia; *Ven*: veneno.) e ilustramos los caracteres específicos diferenciales como ser ojos, órgano estridulatorio, bulbo, palpo y espermatecas. La escala en los dibujos no indica medida sino orientación. Las medidas están dadas en milímetros.

Theraphosa leblondi (Latreille), 1804.

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1805 *Mygale blondii*: Palisot de Beauvois, A.M.F.J., *Insec. rec. Afrique et Amerique 1786-1797*, Paris, p. 4.

1806 *Mygale Blondii*: Latreille P. A., *Genera Crust. et Insect.*, Paris, **1**:83.

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1830 *Mygale Blondii*: Martius F., in *Delect. Anim. Art.*, Monachii, p. 36 (Ven).

1831 *Mygale de Leblond*: Latreille P. A., *Cours d'Entom.*, Paris, p. 506.

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1833 *Mygale blondii*: Perty M., in *Delect. Anim. Art.*, Monachii, p. 271.

1837 *Mygale Blondii*: Koch C. L., *Uebers. Arachn.*, Nürnberg, **I**:35.

1837 *Mygale Blondii*: Walckenaer C. A., *Hist. nat. Ins. Apt.*, Paris, **I**:210 (D ecol.).

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1849 *Mygale Blondii*: Menzel A., *N. jahrsbl. naturf. Ges. Zurich*, **51**:6.

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1863 *Mygale Blondii*: Bates H. W., *The Naturalist on the River Amazons*, London, p. 53, p. 194 (Ecol.).

1864 *Mygale de Leblond*: Simon E., *Hist. nat. Ar.*, Paris, p. 70.

1871 *Theraphosa Blondii*: Ausserer A., *Verh. bot. zool. Ges. Wiener*, **21**:216 (D gen Syn).

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1874 *Theraphosa blondii*: Moggridge J. T., *Trap.-doors Spiders*, London, pp. 188, 234.

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1907 *Theraphosa Leblondi*: Strand E., *Jahresh. Ver. Naturk. Wurt.*, **63**:62 (D ♂ y ♀ Ecol.).

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Diagnosis.

♂ Ejemplar de la colección del Natural History Museum of New York.

Procedencia: Venezuela (Auyan-tepui) a 1.100 m. de altura.

Col. Phelps exp., 6.XII.1937 (El colector la ha dividido por la mitad con un machete).

Largo total: 80. *Cefalotórax*: 40 × 40. *Labio*: 14 de ancho por 7 de alto.

Pata	*Fémur*	*Patela más tibia*	*Metatarso*	*Tarso*	*Total*
I	32	38	24	15	109
II	30	33	24	15	102
III	28,5	32	26	14	100,5
IV	32	36	35	15	113

Cefalotórax igual de ancho que de largo, muy redondeado, su mayor anchura a la altura de la coxa II. *Foseta* apenas recurva, muy profunda, más ancha que el túmulo ocular (6).

Túmulo ocular muy elevado, casi redondo (5 de ancho por 4 de alto).

Fila anterior muy procurva, ojos iguales, casi equidistantes; más grandes que los ojos de la fila posterior. Fila posterior recurva, los MP casi del mismo tamaño que los LP. (Lam. I:6).

Lábio más ancho que alto, con una estrecha banda de espínulas próxima al borde.

Coxas de la pata maxila con el ángulo súpero-interno muy prolongado.

Patas IV, I, II y III muy espinosas. Las anteriores más gruesas que las posteriores. Llaman la atención por el grosor de sus artejos basales. En la pata I el fémur tiene un diámetro dorso-ventral de 9 mm; el II, 8,5; el III, 9 y el fémur IV, 8. Patela más tibia I es un poco mas larga que patela más tibia IV. El fémur IV tiene en su cara posterior una densa escópula de pelos no plumosos. El metatarso IV no tiene escópula.

Órgano estridulatorio. La coxa I en su cara anterior y parte superior, por encima de la sutura, tiene 3 cerdas plumosas de gran tamaño (Lam. I: 5) dispuestas en línea, las que están acompañadas por otras cerdas mucho menores y pelos plumosos que recubren todo el resto de la coxa, trocánter y mas de la mitad basal del fémur. En la coxa de la pata maxila no se observan las cerdas gruesas en su cara externa, pero presenta en su mitad apical espinas negras muy recostadas sobre el artejo, que aumentan de tamaño a medida que se acercan a su extremidad apical. Los pelos plumosos se encuentran en el borde inferior de la cara externa de la coxa, recubren el trocánter y mas de la mitad basal del fémur de la pata maxila.

Pata maxila (Lam. I: 3) *Bulbo*: es casi cilíndrico en toda su extensión siendo excavado en su extremidad apical en forma de cuchara cuyo borde es dentado. (Lam. I: 1 y 2).

♀ de la colección del Museo de Historia Natural de Montevideo.

Procedencia. Venezuela, Dep. Bolívar, Rio Curá.

Col. Pablo San Martín, V. 1957.

Largo total: 84. *Cefalotórax*: 32,5 × 32,5. *Labio*: 8 de ancho × 5 de alto.

Pata	*Fémur*	*Patela más tibia*	*Metatarso*	*Tarso*	*Total*
I	28	34	19	13	94
II	26	30	18	12	86
III	24,5	27,5	20	10	82
IV	27	31	28	11	97

Ejemplar ♀ N.º 2.

Largo total: 76. *Cefalotórax*: 31 × 31.

Pata	*Fémur*	*Patela más tibia*	*Metatarso*	*Tarso*	*Total*
I	27	32	19	12	90
II	25	31	18	11	85
III	23	27	20	11	82
IV	26	31	27	12	96

Ejemplar ♀ N.º 3.

Largo total: 81. Cefalotórax: 34 × 34.

Pata	*Fémur*	*Patela más tibia*	*Metatarso*	*Tarso*	*Total*
I	27	34	20	13	94
II	25	31,5	20	12,5	89
III	24	28,5	21	12	85,5
IV	27,5	32	29	13	101,5

La hembra es en todo igual al macho. El cefalotórax es también igual de ancho que largo en los 5 ejemplares estudiados; tampoco hay diferencias en el órgano estridulatório. La foseta torácica muy profunda, transversa, puede ser más o menos recurva.

Espermateca: Lam. I: 7 y 8. No presenta diferencias en los 5 ejemplares.

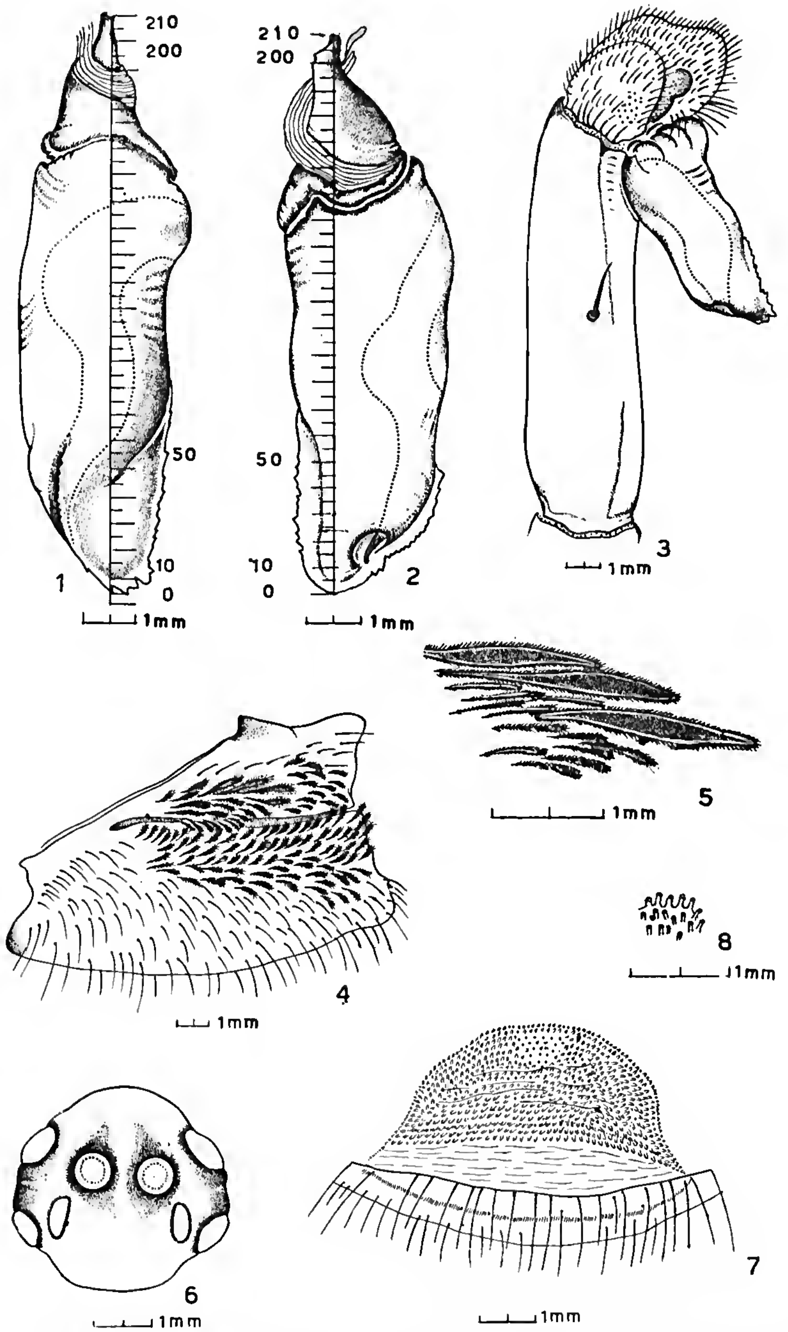
Distribución geográfica. Este género tiene una sola especie, la especie-tipo *Theraphosa leblondi* (Latr., 1804) procedente de las Guayanas (región del Maroni) y de Venezuela. Y a pesar de que Simon niega su presencia en Brasil, dejamos establecido que se encuentra en el Estado de Pará.

Observaciones. Tanto el ejemplar macho como las hembras sobre los que se ha hecho la diagnosis arriba consignada, fueron comparados con los ejemplares típicos de la colección del Muséum National d'Histoire Naturelle de Paris. Dos de las hembras colectadas por P. San Martín quedan depositadas en la colección del Museo Argentino de Ciencias Naturales "B. Rivadavia", como Homeotypus con los números 5874 y 5875.

Summary

With one male specimen from Venezuela belonging to the collection of the American Natural History Museum of New York, and five females collected also in Venezuela by P. San Martín, this species, rare in the museums, has been studied. Much has been written about it in the last century and at the beginning of this one; but since 1936 it has not been mentioned in the spider literature, only in catalogues and lists. In this paper the diagnosis is given pointing out the valid characters to separate it from the other THERAPHOSIDAE. These characters are illustrated in one table with 8 figures. The studied specimens were compared with the Types kept in the Muséum National d'Histoire Naturelle de Paris. For the first time the spermathecae are illustrated. Up to now they were not been described.

Agradecimientos — Agradecemos al Dr. W. H. Gertsch, al Prof. Dr. Max Vachon y a los Señores Pablo San Martín, Roger Arlé y P. Waldir, la gentileza que han tenido al poner el material estudiado a nuestra disposición.



Lam. I: *Theraphosa leblondi*. Fig. 1 — bulbo derecho, cara interna. Fig. 2 — bulbo derecho, cara externa. Fig. 3 — palpo derecho, cara externa. Fig. 4 — coxa I, cara anterior, órgano estridulatorio. Fig. 5 — las 3 grandes cerdas alineadas del órgano estridulatorio. Fig. 6 — ojos vistos de arriba:. Fig. 7 — espermatecas. Fig. 8 — detalle de la pared de la espermateca visto con mayor aumento.

3. ESTUDIO COMPARATIVO DE *PHONEUTRIA FERA* PERTY, 1833 Y *PHONEUTRIA NIGRIVENTER* (KEYSERLING), 1891 (*ARANEA: CTENIDAE*)

RITA D. SCHIAPELLI y BERTA S. GERSCHMAN DE PIKELIN

Museo Argentino de Ciencias Naturales "Bernardino Rivadavia", Buenos Aires, Argentina

Durante largos años el género *Phoneutria* Perty, 1833 figuró en la literatura araneológica como sinónimo de *Ctenus* Walckenaer, 1805.

En 1936, Mello Leitão, en su revisión de la familia CTENIDAE da una clava de géneros y separa a los dos mencionados, principalmente, por la presencia en *Phoneutria* de una densa escópula en la cara interna de las tibias y tarsos de las patas maxilas, tanto en machos como en hembras.

Como género, los ejemplares de *Phoneutria* se determinan fácilmente: pero sus especies están muy confundidas. Son en total unas 12 especies, casi todas del Brasil, de las cuales *Ph. fera* Perty, 1833 y *Ph. nigriventer* (Keys.), 1891 se han citado también para la Argentina. Esas dos especies, de las cuales nos ocupamos en este trabajo, han sido sinonimizadas últimamente por algunos autores; sinonímia que no está bien fundamentada.

Phoneutria fera Perty, 1833 a pesar de haber sido citada para la Argentina por Holmberg y Mello Leitão, no es autóctona; esta especie recolectada por J. B. de Spix en Rio Negro, Brasil ecuatorial, se encuentra en Rio de Janeiro y en litoral de Brasil. A la Argentina llega solamente con los cachos de banana importados de Brasil.

El tipo de *Phoneutria nigriventer* (Keys.), 1891 procede do Rio Grande do Sul, en el Estado del mismo nombre, fué recolectado por von Ihering; esta especie es común en São Paulo y en el Estado del mismo nombre, llegando a la provincia de Misiones, Argentina, en donde se la encuentra con bastante frecuencia.

Para nuestro estudio hemos contado con material de distintas colecciones. Agradecemos al Dr. Max Birabén por habernos facilitado el material de la colección del Museo de La Plata y el de su propia colección; al Dr. Avelino Barrio por habernos entregado para su estudio el material del Instituto Nacional de Microbiología; al Dr. Tomsic del Instituto Miguel Lillo de Tucumán por habernos facilitado algunos ejemplares que poseían en la colección; al Dr. J. de Araujo Feio y a la Dra. Anna Timotheo da Costa por la gentileza de enviarnos en préstamo material del Museo Nacional de Río de Janeiro.

Además contamos con el material depositado en la colección del Museo Argentino de Ciencias Naturales "B. Rivadavia" en donde trabajamos.

Ph. fera y *Ph. nigriventer* son muy semejantes; pero su estudio comparativo nos ha permitido establecer dos caracteres que se corresponden y permiten su

diferenciación; esos caracteres son: la proporción de ancho y largo del epiginio en la hembra y la proporción del ancho y largo de la tibia del palpo en el macho, y el colorido del ejemplar.

Perty, en 1833 hace una descripción muy somera de *Ph. fera* señalando su colorido uniforme.

Keyserling, en 1891 al describir su especie *nigriventer*, que él llama *Ctenus nigriventer*, hace notar que es muy semejante a *fera* de la que se diferencia por el abdomen con manchas dorsales y el vientre con un campo negro; considera que los epiginos de ambas son iguales, cree que *nigriventer* podría ser una subespecie de *fera*; no puntualiza que en su especie el epígino, es más o menos igual de ancho que alto. Que es lo que hace Pickard-Cambridge en 1897 en su "Revisión de la familia CTENIDAE", trabajo que ilustra con dibujos de los epiginos de ambas especies; dibujos que muestran claramente la diferente proporción en los mismos. Esta diferencia la hemos constatado en el material estudiado. (Lam. 1: 1 y 2; 5 y 6).

En cuanto al colorido, *fera* se ha descrito siempre como de abdomen unicolor; en cambio *nigriventer* además de la mancha ventral que le da su nombre, presenta un folio dorsal más claro. Si bien en los machos la mancha ventral negra está a veces muy disminuída, siempre se puede observar una banda obscura en el borde posterior del pliegue epigástrico siendo constante su dibujo dorsal.

Mello Leitão (1936) ilustra los palpos de ambas especies y hace notar la diferencia de proporción de la tibia en ambas; siendo más delgada en *nigriventer* y más gruesa en *fera*.

Vellard (1936) al estudiar el veneno de esas arañas, señala que su acción es muy semejante, no sólo en *fera* y *nigriventer* sino también en *Ph. rufibarbis*, 1833 cuyo epigino es completamente distinto. El veneno de *fera* actua más intensamente sobre las fibras musculares, produciendo convulsiones más violentas y una parálisis más rápida. El veneno de *nigriventer* tiene una acción hipotensa más marcada. *Ph. rufibarbis* excita menos las secreciones.

La correspondecia de los dos caracteres: proporción del epigino en las hembras y de la tibia del palpo del macho con el colorido general: epigino corto y tibía larga con abdomen de dibujo dorsal; epigino largo y tibia corta con abdomen de colorido uniforme; caracteres constantes en el material estudiado (15 ejemplares de *fera* y 50 de *nigriventer*) y como se pueden apreciar en los dibujos que ilustran este trabajo, nos permiten establecer que las dos especies, *Phoneutria fera* Perty, 1833 y *Phoneutria nigriventer* (Keys.), 1891 son válidas.

Los dibujos están realizados por las autoras con cámara clara y pasados en tinta por la dibunante Sra. Sara Kahanoff con un subsidio otorgado por el Consejo Nacional de Investigaciones Científicas y Técnicas de Buenos Aires.

Se agrega un mapa con la distribución geográfica de las especies basadas en las localidades del material estudiado, y una lista bibliográfica de los trabajos mas útiles en su estudio.

Summary

Phoneutria fera Perty, 1833 and *Phoneutria nigriventer* (Keyserling), 1891 are carefully studied, pointing out the differences and similarities, that are illustrated with drawings with camera lucida made by the authors. The study was made on the material of the collection of the Museo Argentino de Ciencias Naturales "B. Rivadavia", Museo de La Plata, Museo Nacional de Rio de Janeiro, Dr. Max Bira-

ben's private collection and other private collections. The diagnosis preceded by its bibliography is given, and also a map showing their distribution based on the material studied:

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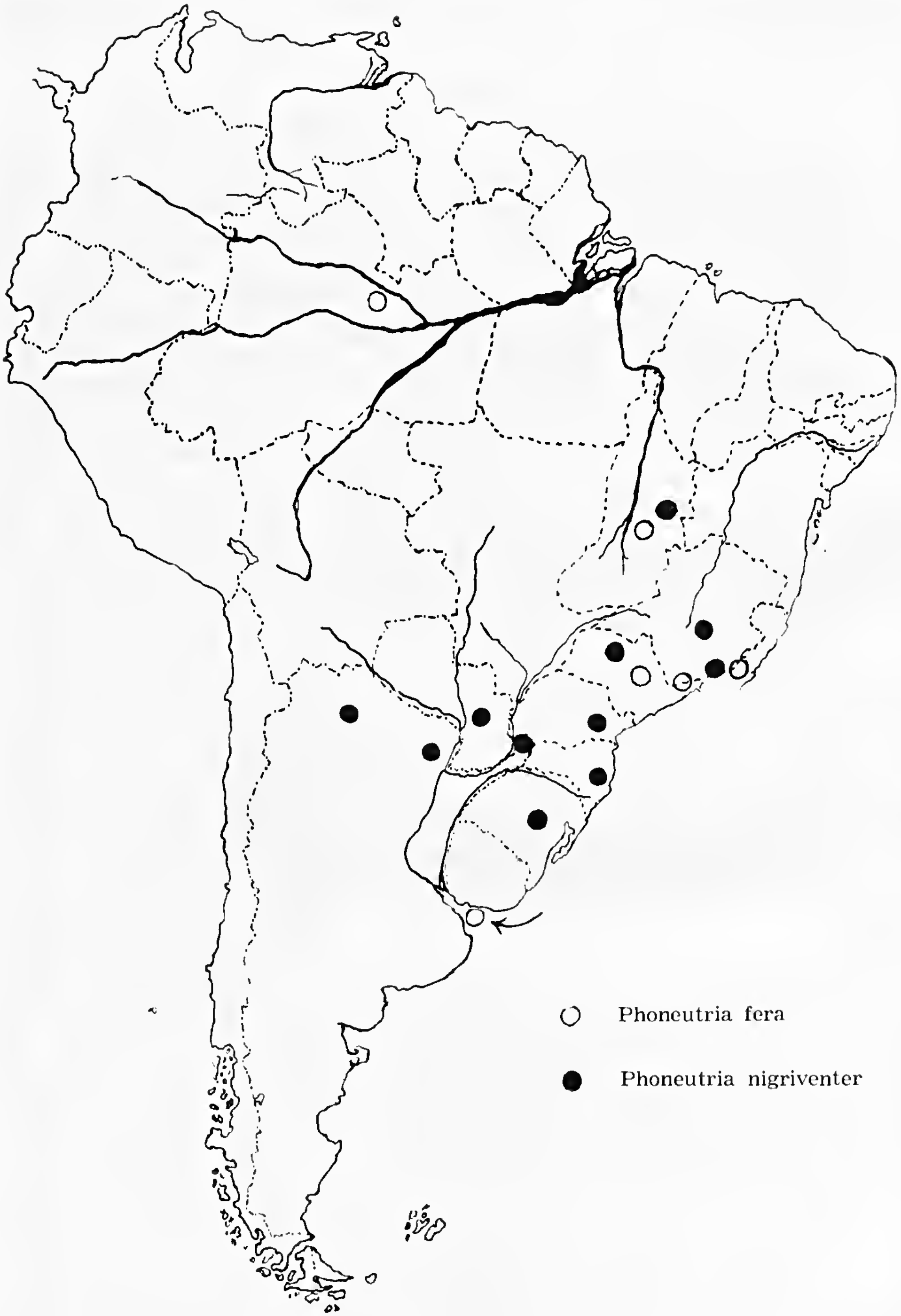
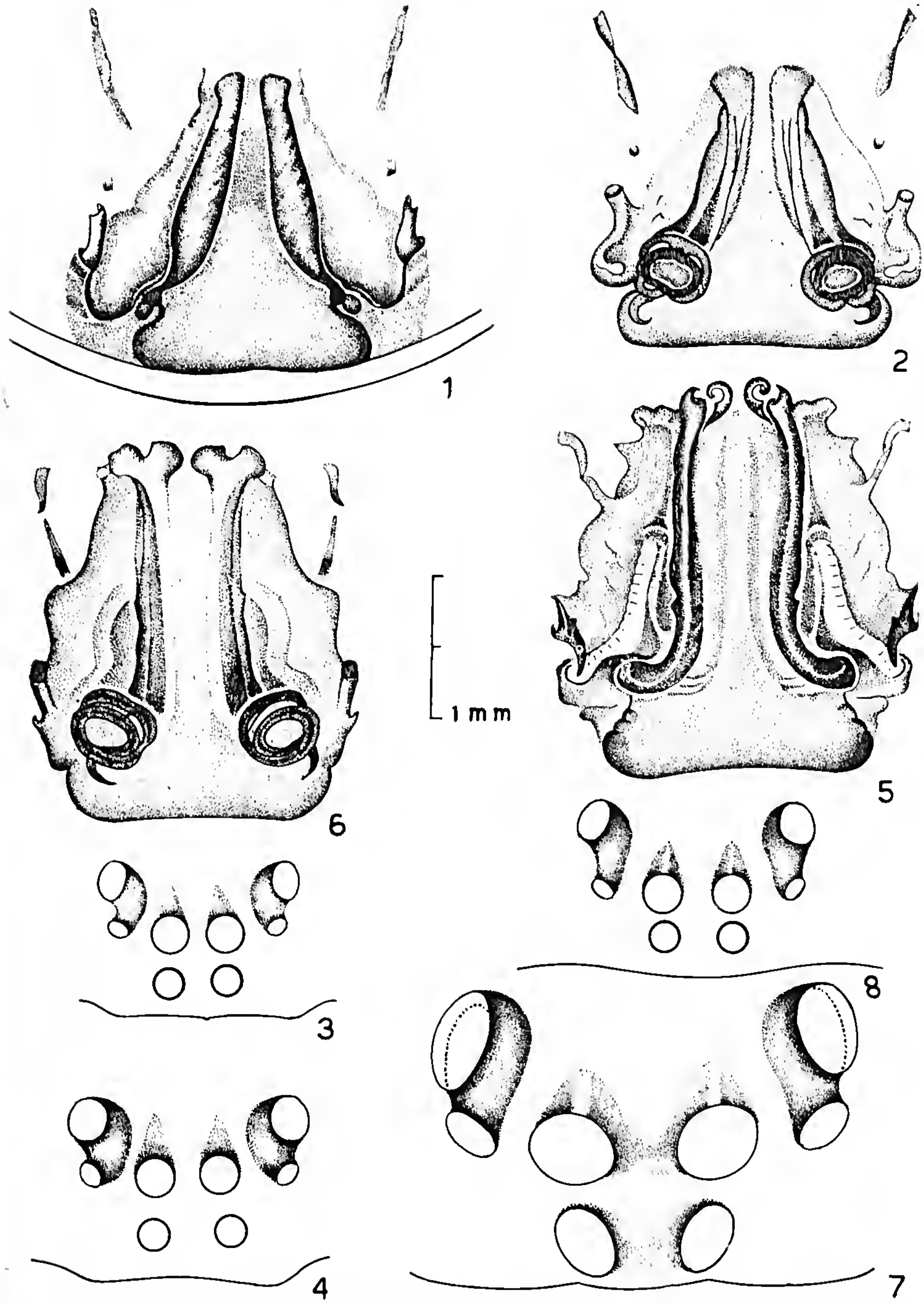
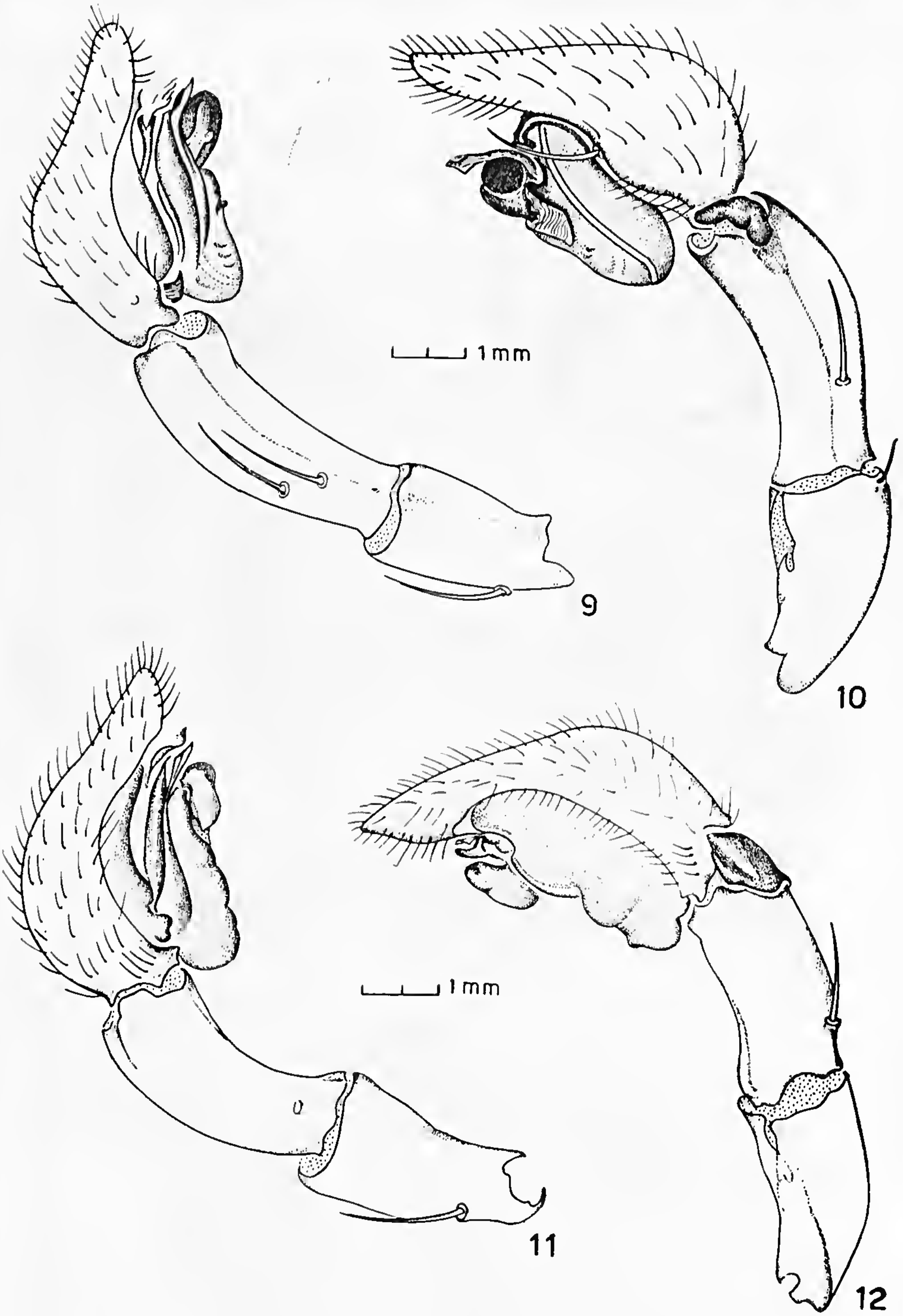

Phoneutria fera
Phoneutria nigriventer



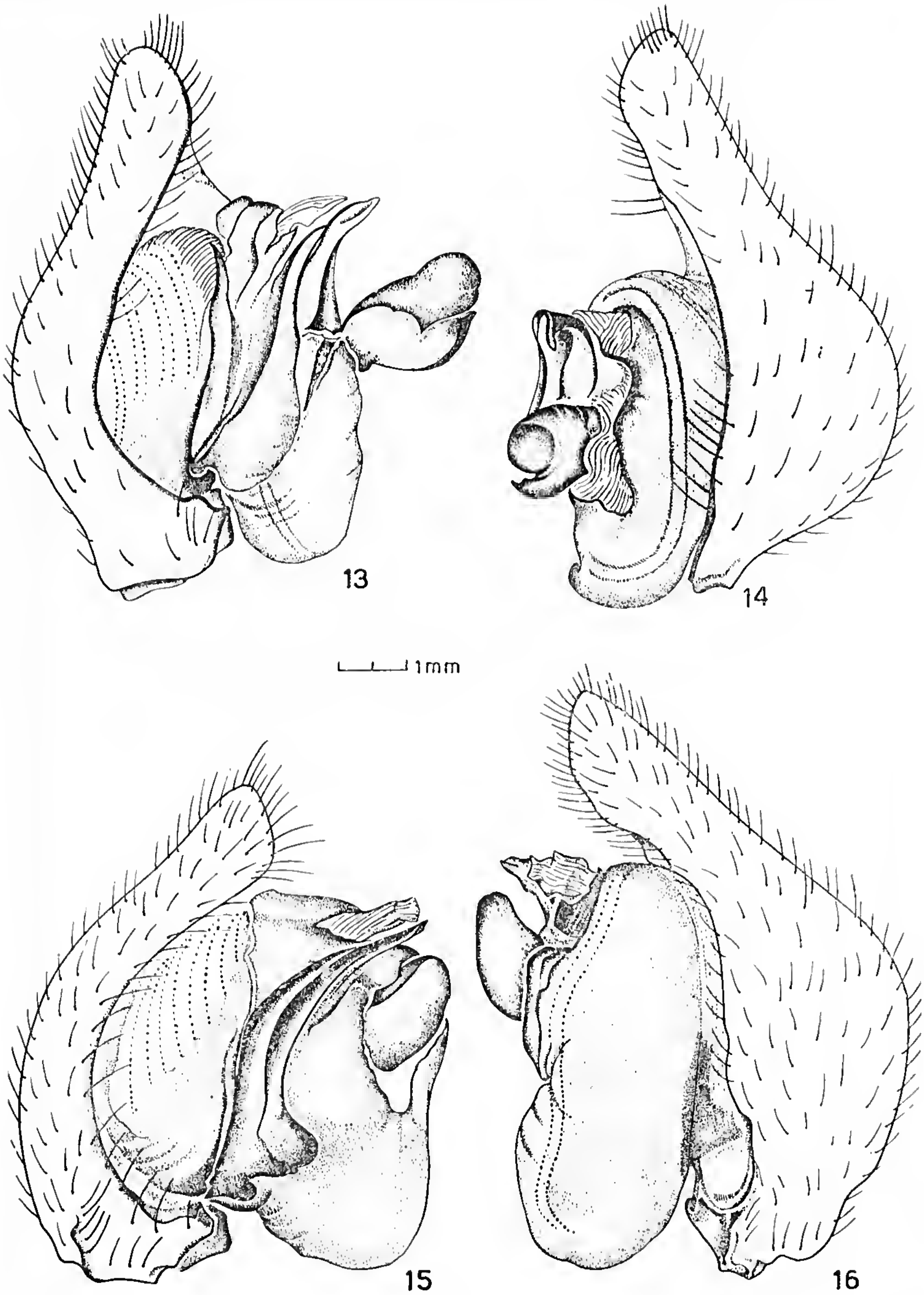
Lam. I: *Phoneutria nigriventer* (Keys.), 1891. Fig. 1 — epigino, cara ventral. Fig. 2 — epigino, cara dorsal (clarificado). Fig. 3 — grupo ocular del macho. Fig. 4 — grupo ocular de la hembra.
Phoneutria fera Perty, 1833. Fig. 5 — epigino, cara ventral. Fig. 6 — epigino, cara dorsal (clarificado). Fig. 7 — grupo ocular del macho. Fig. 8 — grupo ocular de la hembra.



Lam. II: *Phoneutria nigriventer* (Keys.), 1891. Fig. 9 — palpo derecho del macho, cara interna. Fig. 10 — palpo derecho del macho, cara externa.

Phoneutria fera Perty, 1833. Fig. 11 — palpo derecho del macho, cara interna. Fig. 12 — palpo derecho del macho, cara externa.

Mem. Inst. Butantan
Simp. Internac.
33(3):675-682, 1966



Lam. III: Bulbo distendido de *Phoneutria nigriventer* (Keys.), 1891: Fig. 13 — cara interna, Fig. 14 — cara externa. Bulbo distendido de *Phoneutria fera* Perty. 1833: Fig. 15 — cara interna, Fig. 16 — cara externa.

Mem. Inst. Butantan
Simp. Internac.
33(3):683-688, 1966

4. INVESTIGACIÓN ECOLÓGICA SOBRE *LOXOSCELES RUFIPES* (LUCAS), 1834, EN LA REGIÓN COSTERA DEL PERÚ

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INTRODUCÍON

El objeto principal de este trabajo es el análisis de las formas de vida de la araña antropotóxica, *Loxosceles rufipes* en la Província Andina Central, situada en la parte occidental de Sud-América. De esta gran area geográfica que ecológica y fitogeográficamente comprende 3 subprovíncias — Costa, Cordillera y Selva — para el presente estudio elegimos la subprovíncia costera que se extiende desde 30° S (Norte de Chile) hasta 8° S (Norte del Perú), pues es más satisfactorio cubrir una area natural que una política desde que las fronteras de los países son independentes de las barreras naturales que limitam la Flora y Fauna. Señalamos también que todavía este estudio es incompleto y que precisamos realizar investigaciones más acuradas.

MATERIAL Y MÉTODO

La parte principal de este estudio fué realizada durante investigaciones biocenóticas en los principales valles de la costa peruana, donde comprobamos el ecoclima y microclima de este loxoscelineo, tanto en el campo vital rural cuanto en el urbano. Coleetamos material particularmente de los valles de Lima e Ica para su estudio en el laboratorio.

HÁBITAT Y DISTRIBUCIÓN

Loxosceles rufipes se distribuye ampliamente a todo lo largo de los valles de la Costa. Vive preferentemente en el campo vital rural comportándose lapidícola con máxima presencia de alta constancia, pues aquí, de 7.963 araneidos capturados en los nichos ecológicos que deja el pedregal seco y sombreado 69% resultaron identificados como *Loxosceles rufipes* (Lucas), 1834. Con casi igual prevalencia la encontramos debajo de las grandes piedras, terrones de los campos eriáceos, adobes de las ruinas precolombinas, aprovecha también los espacios tubulares de los tallos de la caña brava, *Gynerium sagitatum* de que están construídas las casas rurales. Invade los espacios que dejan los basurales vegetales de gramíneas, cannaceas y otras plantas industriales. No puede sobrevivir dentro del área húmeda de cultivo ni en los biotopos caldeados o mojados del monte ribereño, lomas, zona desértica y zona cultivada. En la zona marina vive en las oquedades que dejan las rocas y grandes piedras de las islas guaneras. A la zona del litoral llega por las playas arenosas con vida vegetal activa, a la desem-

bocadura de los rios en el Océano y a las zonas de fauna cadavérica marina en busca de la gran cantidad de insectos especialmente larvas de coleópteros y dípteros. Menos frecuentemente, en la zona de los desiertos busca albergue en el pedregal protegido por la vegetación xerofítica a donde también llega la entomofauna que utiliza como alimento.

En el campo vital urbano, capturamos 3.646 araneidos y sólo 15% fueron especímenes de *Loxosceles rufipes.* Sin embargo, es oicofílica sinantrópica, pues al no incomodarle la influencia humana invade los edificios modernos. En las habitaciones prefiere los lugares oscuros, abrigados, de poco movimiento y aseo que le garantizan sustraccíon a la vista del hombre durante el día. La búsqueda realizada en el interior de 720 casas tomadas con la técnica del muestreo casual simple en 32 distritos de Lima demonstró que los nichos ecológicos preferenciales son: las azoteas (57%), los rincones oscuros de los sótanos (48%), de las cocinas (42%), de las bibliotecas (12%). Los espacios angulares cercanos al techo o al piso que dejan las molduras de los grandes muebles y cuadros colgados (7%). En los closets, alacenas, cajones y hendidura de las paredes (4 a 5%). En fin esta conducta las lleva también a ocultarse en los espacios interiores que dejan los vestidos colgados en las paredes o en los roperos (3%).

La autoconservación

Alimento — A los biotopos referidos urbanos y rurales acuden también para refugiarse infinidad de **ARTHROPODA** y en los nichos ocupados por este loxoscelinco algunos de entre ellos caen mortalmente por la acción predatora. La constitución del aparato queliceral está admirablemente apropriado para esta actividad coadyuvante de las funciones de nutrición lo cual permite a la araña aprovechar la entomofauna allí existente. Además su tela algodonosa y viscosa sirve como trampa donde quedan selectivamente prendidos artrópodos pequeños particularmente larvas y adultos de dípteros y coleópteros (*Phaleria koepcki* Pic.) y con cierta frecuencia pequeños lepidópteros y ortópteros.

Son voraces; parecen no saciarse succionando las vísceras lisadas de su víctima, pero también experimentalmente los especímenes adultos pueden soportar ayuno hasta en media 16 semanas notándose entoces gran reducción del volumen abdominal.

Durante la luz crepuscular así como en la noche las hembras grávidas se muestran más activas y voraces. Cualquier movimiento las estimula; en cambio las hembras juveniles y los machos continuan al acecho en su madriguera.

Examinando el contenido intestinal microscópicamente a fresco y con coloración de Lugol y Gram encontramos detritos indeterminables y una que otra vez músculos estriados. Casi siempre pudimos detectar la presencia de *PROTOZOA* (rizopodos y flagelados), bacterias (cocos y bacilos), hongos (levaduras).

La locomoción dentro de su hábitat o para ampliar su campo vital es enormemente facilitada por la plasticidad de sus patas que se adaptan a los laberintos del pedregal y basural ocupando mínimo espacio. Al avisorar el peligro son veloces, adquieren impresión vivaz. Se empinan para correr sobre superficies horizontales y parece que amplian la base de sustentación arrastrando casi la superficie ventral corporal cuando trepan superficies verticales. Evitan caminar contra la gravedad pues no puden utilizar la substancia serígena de sus hileras como paracaídas.

Resistencia: medio físico — Este loxoscelineo es muy sensible a la excesiva humedad; el exoesqueleto parece fallar contra la acción lítica del agua. El abdomen es el más vulnerable pues se moja con gran facilidad debido a que es pubescente y guarda mayor tiempo el agua permitiendo el desarrollo de hongos; por esto muere cuando se riega el biotopo que ocupa.

Huye penosamente y a veces sin éxito de los pedregales que quedan enlodados por el polvo y llovizna. Logra salir de los muy caldeados por el sol estival; es entoces que suelen correr de día procurando otro biotopo sombreado cercano a la vegetación y es así que pasan de las azoteas hacia las habitaciones del hombre. Cuando huye se desplazan a 0,95 m/seg. en media.

El biotopo sombreado puede calentarse por encima de 60°C en las horas de fuerte insolación o enfriarse bruscamente a 4°C al declinar el sol. Sin embargo parece que su microhabitat amaina estas transiciones creando un microclima favorable; además *Loxosceles rufipes* es euriterma con lo cual logra condiciones óptimas en el biotopo sombreado durante el verano. Aqui permanece inmóvil aún con una humedad atmosférica relativa de 40 a 70% en el aire que está en contacto con la superficie del piso.

Experimentalmente soporta 6 horas en media a la sequedad de la estufa a 37°C. Vive en condiciones óptimas a 17 y 21°C. Las temperaturas de 26 a 30°C la matan en 12 horas. Puede vivir sin recibir agua durante 12 semanas lo que acontece cuando está en cautividad. El riego de sua biotopo es un factor desfavorable aun durante el verano.

Resistencia: medio biológico — Las hormigas y los véspidos (*Polistes sp.*) son los principales enemigos específicos. Los animales de pastoreo mueven el pedregal y el hombre limpiando y quemando los abrojos alteran el biotopo.

Los Protozoarios, hongos y bacterias encontrados en el aparato digestivo evidentemente son parásitos, cuya araneidopatogenicidad e identificación taxonómica ignoramos.

En la competencia con otros araneidos ejerce acción dominadora ganando el biotopo a arañas de su misma talla o menores, siendo por esto especie representativa del basural vegetal y pedregal.

Algunas especies de FORMICIDAE suelen invadir momentaneamente en horas del día el biotopo de *Loxosceles rufipes* para apoderarse de las ooteeas. En breves instantes se apoderan de los huevos en presencia de la propia madre. que no efectúa resistencia alguna contra esta agresión.

Los individuos adultos tienen coloración simpática con el terreno polvoriento de color marrón con todos sus matices. Los individuos jóvenes tienen de modo general coloración que vira del amarillo claro citrino al castaño oscuro ferruginoso. Así, los estadios juveniles parecen protegidos por el color contra la predación de véspidos (*Polistes*, *Eumenes*), sólo si permanecen inmóviles, pues basta que realicen la fuga para que invariablemente caigan víctimas del hymenóptero. Raras veces pueden escapar cuando se las coge por los apéndices, de los cuales se desprenden por autotomía. Los apendices desprendidos continúan rápido movimiento, distrayendo de este modo al predator, que puede ser un pájaro de pequeño tamaño o una lagartija (*Tropidurus peruvianus*).

En el mismo biotopo gana la competencia alimentaria a múltiples **ARACHNIDA**; sin embargo la pierde cuando existen en abundancia nidos de **HYMENOPTERA** y/o **SCORPIONIDA** (*Hadruroides lunatus*, *Brachistosternus ehrembergi*), en sus vecindades, pues estos son enemigos ocasionales.

Resistencia: medio social — Los individuos que no han alcanzado madurez sexual, en presencia de abundante alimento comparten el nicho ecológico; así pudimos encontrar ocupando espacios contiguos intercomunicados y bajo una misma piedra hasta 19 individuos próximos a la muda que les confirió adultez.

Los adultos son antisociales, sin embargo, pueden tolerar la presencia momentanea de estadios juveniles de su misma especie tanto en su medio natural como cuando están en cantividad.

Los individuos del mismo estadio son siempre canibales; el individuo mejor constituido mata a su rival mediante el aparato queliceral.

Cuando los individuos han logrado madurez, su coloración aumenta de tonalidad, se tornan absolutamente antisociales iniciando entonces una nueva búsqueda de nichos ecológicos.Entran en competencia con otros **ARTHROPODA** también lapidícolas a los cuales no solo les quita campo vital sino que los usa como alimento. El individuo adulto que ha logrado total desarrollo se encuentra mejor protegido por su corpulencia y por la acción mortífera de su aparato queliceral. Probablemente por esta razón los pequenos pájaros que se alimentan de artrópodos y la lagartija (*T. peruvianus*) que son enemigos ocasionales de *Loxosceles rufipes*, prefieren dar caza a los estadios juveniles.

La conservación de la especie

Las hembras a su cuarta muda, excluyendo la que se efectúa intraootecalmente, devienen aptas para las funciones de reproducción, en tanto que los machos se muestran sexualmente aptos a la tercera. Los especímenes adultos con casi 51 semanas de edad muestran marcado dimorfismo sexual. El macho es de cuerpo gracil, de patas más largas, palpos plegables de morfología característica especialmente en el bulbo y embolo adaptables a los receptáculos seminales de la hembra que también exhiben morfología de valor taxonómico específico. La estructura morfológica de los palpos sirve para el transporte de los espermatóforos e inoculación durante el acoplamiento que generalmente es nocturno.

La hembra grávida tórnase voraz e irritable, llegando al máximo de agresividad y canibalismo. No permite la presencia de sus semejantes de la misma cronología o de otros individuos en su biotopo, sin embargo muéstrase inerme para impedir la visita de las hormigas que llegan para examinar las ootecas, que las invaden si no están bien construídas.

Durante la oviposición, que sospechamos se deba efectuar a los 2-3 meses del apareamiento, la hembra se queda dentro del saco ovígero, que es laxo y que previamente fué tejido por ella. El saco ovígero que suele contener de 40 a 150 huevos embrionados es revestido, por una capa de tela algodonosa y laxamente asegurado a los accidentes de su madriguera. La oviposicion puede durar de una a dos semanas y por lo general construyen una sola ooteca, habiendo casos que llega a fabricar hasta 3, otros casos en que la ooteca no tiene huevos o estos no son viables.

La eclosión de los huevos depende de la temperatura, por esto es más frecuente en la estación cálida y dentro de las habitaciones abrigadas. Durante todo el año en los biotopos naturales referidos se puede encontrar todos los estadios del ciclo evolutivo en correlación positiva a la variabilidade climática que se da en la Costa. Además dentro de las casas tienen microclima independiente de la estación del año dentro de un margen relativo.

En su medio rural así como en cantividad la hembra puérpera no prodiga cuidados a las crias; sin embargo en ausencia de alimento demuestra su instinto maternal absteniéndose del canibalismo con su prole. El macho tampoco tiene participación alguna en el cuidado de los vástagos.

Las arañitas recién eclosionadas al cabo de 4 o 5 días realizan su primera muda y abandonan la ooteca. Desde este momento empiezan a cumplir las funciones biológicas básicas de la autoconservación.

En su medio natural se dispersan en pos de alimento en los espacios que dejan el pedregal, basural y espacios protegidos de las habitaciones donde encuentran larvas de coleópteros y dípteros que utilizan como alimento. En cautividad reciben *Drosophila sp.*. ninfas de **ORTHOPTERA** (*Blata* y *Periplaneta*), larvas de MUSCIDAE (*Musca*, *Destrus*, *Stomoxis*) y larvas de **COLEOPTERA** (*Phaleria sp.*) a las cuales atacan para luego succionar las vísceras licuadas por los jugos digestivos que inyectan.

Biocenosis

Las formas de vida que hemos señalado son adaptaciones de este loxoscelineo a los espacíos protegidos y oscuros que le ofrece el campo de vida rural y urbano y a las comunidades vitales lapidícolas.

En fin, el comportamiento y complejos estructurales de esta araña antropotóxica, la señalan como muy apropiada para vivir en las viviendas del hombre de la misma manera que en los biotopos del pedregal y basural vegetal secos próximos a terrenos con vegetación del medio ambiente biótico rural.

5. MI EXPERIENCIA SOBRE LOXOSCELISMO

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El objetivo de este trabajo es analizar el cuadro clínico producido por la picadura de la araña de los rincones, *Loxosceles laeta* *, a través de 200 casos de nuestra casuística, tomados al azar, ocurridos en Valparaíso y pueblos vecinos (Chile), entre 1956 y 1966.

Los accidentes causados por la introducción de ponzoñas de arañas en el hombre, siguiendo el camino natural de picadura a través de la piel, están condicionados por el tipo del veneno, la época del año, el estado de la araña, la cantidad de ponzoña disponible, el lugar por donde es introducido, el camino seguido por el tóxico y la resistencia del paciente (5).

El accidente ocurre cuando las arañas son martirizadas inadvertidamente. De ordinario no acometen, sólo se defienden. En nuestra casuística la picadura se produjo cuando el afectado se vestía o mientras estaba en cama durmiendo.

La época del año en que sucede el accidente tiene importancia porque durante el verano las arañas están más vivaces y el calor, alcalinizando las ponzoñas, las hace más activas (6). De nuestros 200 casos, 98 acaecieron durante el verano (49%). En cambio, en invierno se registraron solamente 31 casos (15.5%). En primavera hubo 42 casos (21%) y en otoño 29 (14.5%).

El lugar de la piel por donde es introducido el tóxico es también importante. Las zonas de piel fina o las regiones muy irrigadas son las que determinan los cuadros clínicos más serios.

En cuanto a resistencia humana, es factor que debe considerarse. Los niños son los que hacen más graves casos de aracnidismo.

El veneno del *Loxosceles laeta* es dermotóxico y en algunos casos dermoviscerotóxico.

La picadura de la araña de los rincones es capaz de engendrar, entonces, dos cuadros clínicos diferentes: — Uno localizado, benigno o de mediana gravedad, llamado loxoscelismo cutáneo o "mancha gangrenosa" y, otro generalizado, muy grave y mortal con frecuencia, el loxoscelismo cutaneovisceral. — En el primero, el veneno actúa sólo localmente. En el segundo, difundido, obra sobre todo el organismo y afecta en especial la sangre, el hígado y los riñones.

Cualquiera está expuesto a ser picado por esta araña que es común en las viviendas, aún en las muy aseadas y cuidadas. En nuestra casuística hubo 97 casos de mujeres (48.5%) y 103 de hombres (51.5%).

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* Continuamos usando el término *"laeta"* y no *"rufipes"* hasta que los especialistas en sistemática se pongan de acuerdo acerca de cual debe perdurar (1, 2, 3, 4).

La zona afectada del cuerpo puede ser cualquiera. Con todo, el mayor número de casos se refiere a las extremidades, el cuello y la cara. De nuestros 200 casos, 139 fueron de las extremidades (69.5%), con predominio en los antebrazos, 28 casos (14%) y en los muslos, 21 casos (10.5%), en el cuello y nuca 18 (9%) y en la cara 21 (10.5%). En el tórax tuvimos 15 casos (7.5%), en el abdomen 4 (2%) y en los genitales 3 (1.5%).

En materia de edad, el mayor número de afectados tenía entre 16 y 50 años, es decir 135 casos, o sea, el 67.5%. El menor tenía un mes y el mayor sobre 70 años.

Producido el empozoñamiento, en las primeras veinticuatro horas no se puede predecir lo que va a sobrevenir. El comienzo es igual en cualquiera de las dos formas de este aracnidismo.

Se inicia con el dolor de un pinchazo. Sin embargo, en algunas oportunidades, los afectados no recuerdan el incidente inicial. De nuestros 200 pacientes, 158 sintieron el pinchazo (79%). La picadura sirve para que el afectado busque la causa y, muchas veces, encuentre la araña y la destruya. De nuestros enfermos no la vieron en 48 casos (24%), la vieron 149 (74.5%), la destruyeron 77 (38.5%), se pudo identificar en 36 casos (18%). En todos ellos era *Loxosceles laeta.*

Al lancetazo sigue un período asintomático o de latencia, de ordinario corto, de 5 a 10 minutos, pero que a veces puede llegar hasta 8, 10 y 24 horas.

Estalla, entonces, un dolor local creciente, con sensación de quemadura, y unas pocas veces con prurito, dolor que puede adquirir caracteres impresionantes y cuya pertinacia e intensidad, en cualquier caso, mortifica mucho. En nuestros casos, el dolor local se hizo presente en 193 (96.5%), acompañado de sensación de quemadura en 180 (90%), con prurito en sólo 22 casos (11%).

La piel, en el sitio afectado, enrojece por congestión de los capilares, se edematiza y se pone tensa y dura.

En este momento el cuadro clínico se individualiza en una de las dos formas antes señaladas. Va al proceso cutáneo o se generaliza y se hace visceral. Por lo común esto sucede dentro de las primeras veinticuatro horas.

LOXOSCELISMO CUTANEO O MANCHA GANGRENOSA

En la forma cutánea no hay o es limitado el compromiso del estado general, en cambio se produce una lesión local de variables proporciones, muchas veces de alarmante extensión y de difícil reparación.

El dolor local va aumentando y, se bien es cierto en algunos casos es tolerable, en otros puede llegar a adquirir tal violencia y pertinacia que más de algún paciente ha deseado que le amputasen la extremidad afectada para librarse de él o en su desesperación ha pensado en suicidarse. El dolor es permanente, con exacerbaciones periódicas. Limita los movimientos, crea impotencia funcional transitoria, causa insomnio y desasosiego. Tiene la ya citada característica tan desagradable y típica de la sensación de quemadura desesperante.

Desde las primeras horas aparece, en el sitio de la picadura, una mancha de tamaño variable, de color rojo vinoso, en cuyo centro es, a veces, posible distinguir los puntos por donde penetraron los quelíceros de la araña. Alrededor de las veinticuatro horas el centro de la lesión se pone violáceo y después negro. En su contorno se diseñan zonas pálidas, blancas y otras moradas, como vetas

irradiadas irregularmente, todo lo cual recuerda el color del mármol jaspeado. de allí el nombre que le damos de "placa marmórea". Depende de una isquemia local y de hemorrágia con induración. Como un halo, en torno de ella, la piel no directamente afectada. desde un comienzo roja. acentúa su color, con gran vasodilatación.

Típica placa marmórea vimos en 173 de nuestros casos (86.5%), algunas pequeñas y otras de tamaño impresionante.

Surgen sobre esta placa y, a veces antes que ella se forme o sin que se forme, en tiempo variable de pocas horas, flictenas, a veces gigantescas, de contenido seroso o sero-hemático, asépticas por cierto. En algunas circunstancias la vesícula es única y en oportunidades constituye la exclusiva manifestación de la picadura de la araña. Tuvimos flictenas en 80 casos (40%).

El edema que se insinúa desde el principio, se va extendiendo a vastas superficies del cuerpo, sobre todo en las zonas declives. Es consistente, regular. Bajo la placa marmórea se hace más duro, sitio donde se produce gran infiltración. En 162 de nuestros casos fue notorio (81%).

En la placa marmórea y sobre todo en la parte negra de ella desaparecen la sensibilidad al dolor y térmica. Mientras tanto que en la región roja periférica la sensibilidade es exquisita.

El tamaño de la placa marmórea es variable. Nosotros tuvimos casos en que medía desde uno hasta treinta y más centímetros de diámetro.

Entre el 5.º y el 6.º día después de la picadura, han disminuído el edema y el rubor, mientras que la placa marmórea se pone negra. Se deslinda una escara apergaminada y seca, dura y algo brillante. cuyo tamaño guarda relación con las dimensiones de la placa, escara que delimitándose bien. se va soltando por los bordes hasta caer a la tercera semana (5, 7, 8).

El aspecto de gangrena seca que presenta la lesión y su color negro le han significado de denominación. desde hace más de un siglo, de "mancha gangrenosa" (9, 10, 11). En nuestra casuística hubo necrosis de importancia en 78 casos (39%).

Cuando se desprende la escara queda una úlcera de superficie rosada, mamelonada. irregular, anfractuosa, resumando un líquido amarillento y sangrando con mucha facilidad.

En los casos en que la piel está directamente aplicada sobre las aponeurosis musculares, éstas se ven en el fondo de la úlcera.

Es interesante señalar que con el deslinde de la escara y la disminución del edema, también menguan los dolores y por fin cesan del todo. El desprendimiento de la escara es indoloro, lo que acontece, como lo hemos dicho, hacia la tercera semana (7, 8, 12).

Es menester hacer hincapié en que lo que en el 96,5% de nuestros casos desesperaba al paciente era el dolor local, la sensación de quemadura y el insomnio que, naturalmente, resultaba su consecuencia.

La adenopatía que se registra, en oportunidades, se debe a infecciones piógenas sobreagregadas. La presentaron 27 de nuestros casos (13.5%).

La reparación de los tejidos es muy lenta. En lesiones extensas es menester hacer injertos cutáneos. El tiempo que ha demorado la curación total ha sido, en general de 20 a 30 días, pero tenemos casos de 5 a 8 días y otros hasta de 116 días.

El proceso deja una cicatriz irregular, parda o azuleja. a veces queloídea y retráctil, excepcionalmente dolorosa.

Loxosceles laeta.

Loxoscelismo — El edema.

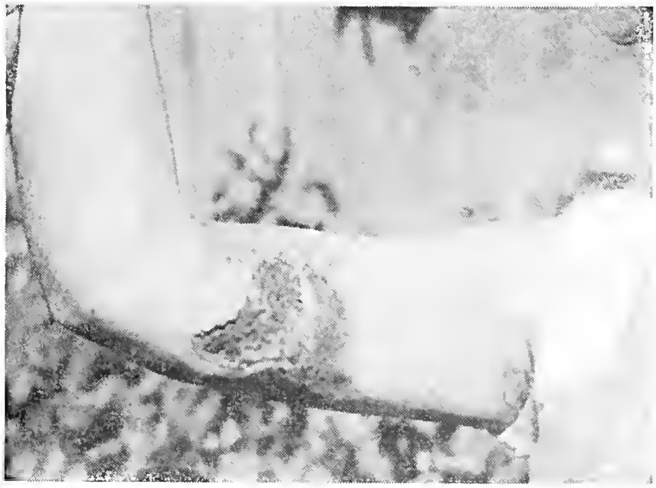
El curso de esta forma de loxoscelismo es favorable siempre. Los problemas surgen de las incapacidades para el trabajo, por suerte de tipo transitorio o en casos muy raros de carácter permanente, derivadas de la larga evolución hasta la mejoría, de la ubicación de la lesión, de la cicatriz y de la retracción de los tejidos que puedan limitar los movimientos de las extremidades o del cuello, o bien de la secuela de cicatrices deformantes de la cara.

En el loxoscelismo cutáneo hay nulo, escaso o mediano compromiso del estado general del paciente, completamente independiente del carácter, tamaño e importancia de la lesión local.

Consideramos en el loxoscelismo, clínicamente hablando, cuatro grupos de pacientes: 1.° — Sólo con lesiones locales, sin ningún compromiso del estado

Flictenas.

La mancha gangrenosa.



La úlcera.

general. 2.º — Aquellos con ligero compromiso. 3.º — Con mediano compromiso. 4.º — El grave proceso cutáneo-visceral.

Los tres primeros grupos corresponden al loxoscelismo cutáneo. El cuarto forma la entidade cutáneo-visceral.

Del primero, con sólo lesiones locales, sin ningún compromiso del estado general, de los 200 casos, tuvimos 108 (54%).

Llamamos con ligero compromiso a los que además de las lesiones locales presentaron cefaleas y fiebre. Contamos con 39 casos (19.5%).

Contemplamos como de mediano compromiso a aquellos que presentaron, fuera de las lesiones locales, fiebre, cefaleas, vertigos, nauseas, vómitos, diarrea, sudores y crisis nerviosas. Tuvimos 31 casos, en este grupo (15.5%). Excepcionalmente hay prurito o erupción cutánea morbiliforme. Ello sucedió en 19 casos (9.5%).

En cualesquiera de los citados casos el pulso se acelera en relación con la temperatura; las presiones se mantienen; la uremia y la glicemia no se modifican; las pruebas de función hepática, los tiempos de sangría y de coagulación y la resistencia globular son normales. Igualmente el hemograma, sedimentación, orina y electroeardiograma no cambian.

Finalmente agrupamos en el último conjunto los casos graves, es decir la entidad clínica que lamamos loxoscelismo cutáneo-visceral, de impresionante violencia, con una lesión local mínima y un compromiso del estado general fulminante con anemia hemolítica, necrosis hepática y nefrosis hemoglobinúriea. De estos tuvimos 22 casos, es decir el 11% del total, com 7 muertes (3.5% de los 200 casos y 31.81% del loxoscelismo cutáneo-visceral).

LOXOSCELISMO CUTÁNEO-VISCERAL

Clínica — En la forma clínica cutáneo-visceral del loxoscelismo la gravedad es mucha y el pronóstico sombrío.

La lesión local, las más de las vezes, pasa a segundo término, porque la violencia y la rapidez del proceso casi no le dan tiempo para formarse y, en aquellos pacientes que mueren rápidamente, apenas hay una mancha rojo-cianótica, edema y un esbozo de placa marmórea. El dolor quemante, en cambio, no falta nunca.

Contrastando con esta condición el compromiso del estado general es impresionante. Hay intenso malestar general, desasosiego, cefaleas y fiebre. Del grupo de 22 casos cutáneo-viscerales que tuvimos dentro de los 200 de loxoscelismo, los 22 presentaron malestar general, 15 desasosiego, 16 cefaleas y 20 fiebre. En muchos de ellos sobrevinieron vértigos, taquicardia, hipotensión y en un fuerte número, 13 de los citados, hubo náuseas, vómitos con frecuencia con sangre. Alrededor de las 12 a 24 horas surgen los síntomas más alarmantes: Equímosis, ictericia y hematuria y hemoglobinuria. En nuestros 22 casos 5 hicieron equímosis en diferentes partes del cuerpo; 15 ictericia y 19 hematuria. Horas más tarde aumentan las cefaleas, vienen calofríos y decaimiento, desaparece el sueño y mientras la taquicardia acrece la hipertermia alcanza a 40° centígrado, las presiones descienden y la piel se pone terrosa.

Como se vaya agravando el cuadro clínico irrumpen acentuadísimas dispnea y cianosis y se marcan más la ictericia y las hemorragias imponiéndose la anemia. En muchos pacientes aparecen signos de congestión y edema pulmonar.

Por fin la respiración se hace estertorosa; tos y desgarro muco-espumoso y sanguinolento ahogan al enfermo; el pulso se pone filiforme, las presiones descienden más aún, se suprime la diuresis, el desasosiego se extrema y el enfermo entra en coma hasta que muere.

Pruebas de Laboratorio — Las pruebas de laboratorio nos ofrecen una serie de hechos muy importantes.

En la sangre enseñan una anemia de tipo hemolítico. Hay formidable destrucción de los hematíes. En uno de nuestros casos se contaron sólo 840 000 por milímetro cúbico. Le acompañan trombocitopenia, y reducción del hematocrito (en uno de nuestros pacientes no alcanzaba a 7%). En la serie blanca irrumpe una fuerte leucocitosis, verdadera reacción leucemoíde con 30, 40 y más de 50 000 leucocitos por milímetro cúbico, con desviación a la izquierda de la fórmula, en algunos casos com 31 segmentados y 47 baciliformes. La sedimentación, que en el loxoscelismo cutáneo es normal, sube en la forma visceral a más de 50 mm en la primera hora. La uremia sube también. En uno de nuestros casos llegó a 5 gramos por mil. La glicemia al principio alza transitoriamente y luego queda en hipoglicemia. Hay hiperbilirrubinemia y hemoglobinemia.

En la orina, la densidade baja (1009 y 1010), aparecen albuminuria, hematuria, hemoglobinuria y cilindruria.

Los casos más graves terminan en la muerte, entre las 30 y las 48 horas después de la picadura de la araña. En nuestra casuística, de los 22 casos de loxoscelismo cutáneo-visceral, tuvimos 7 mortales, es decir, 31.81% de los 22 y 3.5% de los 200.

Anatomía patológica — Los hallazgos coinciden con lo que otros médicos y nosotros mismos hemos observado en los experimentos de laboratorio, con animales sometidos a la acción de la ponzoña de esta araña (5, 7, 8, 12, 13, 14).

Localmente se produce edema sero-sanguinolento; los tegumentos se infiltran; el proceso puede extenderse muchísimo por vecindad, comprometiendo hasta los músculos regionales. En el dermis hay fuerte dilatación capilar con perivascularitis. Las lesiones típicas de la placa necrótica rara vez se ven en las autopsias porque la muerte sobreviene antes de que puedan formarse. El resto de la piel aparece pálida y con ictericia.

En la sangre, la ponzoña determina hemolisis intravascular, con las correspondientes hemoglobinemia y hemoglobinuria, con lesión renal ulterior, la nefrosis tóxica aguda o hemoglobinúrica. Con la liberación excesiva de hemoglobina aumenta la concentración sérica de bilirrubina y aparece la ictericia. Como consecuencia se produce una hemosiderosis generalizada. El revestimiento endotelial de los vasos sanguíneos se va tiñendo de hemoglobina. La excreción de los pigmentos de ella termina por produzir la ya citada nefrosis hemoglobinúrica.

En los casos más graves sobrevienen cambios grasos en el corazón, riñón e hígado. Pero, no debemos olvidar que los agentes tóxicos no sólo actúan sobre la sangre sino que también causan simultáneamente lesiones graves en otros órganos que a veces llegan a predominar.

En muchos casos hay trombocitopenia. Entonces el tiempo de coagulación resta normal, pero se alarga el tiempo de sangría. En algunos enfermos hemos tenido tendencia a las hemorragias de las mucosas y en la piel, con grandes equímosis. Esto refleja una acción del tóxico sobre la médula ósea.

Siendo el hígado uno de los principales centros de desintoxicación que posee el organismo, los venenos actúan sobre él. En los casos benignos, sólo hay tumefacción turbia de las células hepáticas. En lesiones más graves hay infiltración grasa y en las peores circunstancias destrucción o necrosis en focos o

difusa, además de hemosiderosis. En nuestros casos fatales había focos de necrosis, moderada disociación travecular, difusa infiltración grasa y hemosiderosis. (Las células de Kupfer tumefactas y el citoplasma hemoglobínico, además de nódulos de hierro en los espacios intertraveculares).

Los riñones experimentan con violencia la acción de la ponzoña. La parte más vulnerable de ellos son los tubos contorneados, en su parte proximal, que sufren tumefacción, metamorfosis grasa y aún necrosis, según la gravedad del caso. Los glomérulos y tubos colectores resisten más.

Las alteraciones renales comienzan antes de las 24 horas. En la orina rápidamente aparecen cilindros granulosos y pigmentados con hemoglobina. Los tubos se llenan de cilindros y sobreviene necrosis y degeneración de los epitelios. El cuadro, en todos nuestros casos de loxoscelismo cutaneo-visceral fue de nefrosis hemoglobinúrica. En ellos había edema, hemorragias en los riñones, túbulos llenos de cilindros hemáticos y paredes tumefactas. Tanto la pared de los túbulos como la de la cápsula de Bowman aparecían cargadas de hemoglobina y la cápsula llena de glóbulos rojos y hemoglobina. En la vejiga encontramos orina con eritrocitos y hemoglobina y sus paredes embebidas de hemoglobina.

En la médula ósea hallamos predominio de elementos primitivos, mielocitos, granulocitos macrófagos, eosinófilos y escasos plasmocitos.

En los pulmones encontramos acentuado edema, hiperemia y hemorragias. Igualmente se compromete la pleura y termina ésto en hemotórax. Las arteriolas están llenas de sangre hemolizada.

En el corazón, en algunos casos encontramos degeneración grasosa y tumefacción turbia de las fibras musculares, además de hemopericardio.

En el cerebro, la reacción a la ponzoña se parece a lo que ocurre con algunos otros tóxicos, encontramos edema, hiperemia, perivascularitis y piqueteado hemorrágico.

En el resto de los órganos había hiperemia, edema, foquitos hemorrágicos (suprarenales y bazo) o bien edema y erosiones (tubo digestivo).

Diagnóstico — Clínicamente es posible hacer el diagnóstico. Ni la placa marmórea, ni la escara pueden confundirse. Con relativa frecuencia el paciente no ha visto la araña y no se sabe cómo se ha iniciado su enfermedad, pero el dolor quemante, la placa marmórea, las flictenas, la mancha gangrenosa, sin compromiso o muy escaso del estado general, la mayor parte de las veces sin ganglios afectados, llevan al diagnóstico de loxoscelismo cutáneo.

En la forma cutáneo-visceral, la lesión local es poco ostensible, pero existe y, acompañándose de fulminante agravamiento del estado general, con fiebre, ictericia, hematuria y hemoglobinuria no es un problema llegar al diagnóstico.

Naturalmente, en la forma cutánea hay que distinguir de carbunclo, forúnculos y picaduras de insectos. En la forma visceral, de aquellas enfermedades con ictericia y hemólisis.

Pronóstico — Antes de las primeras 24 horas no es posible predecir la evolución. Pasadas éstas, en el caso del loxoscelismo cutáneo no hay peligro para la vida pero, la curación puede demorar meses y dejar cicatrices dolorosas y aún impotencia funcional de los miembros si la lesión se produjo en las extremidades. Tenemos un caso en que a pesar de los injertos de piel que se le hicieron, por la extensión de la pérdida de piel que sufrió en el muslo izquierdo, demoró 116 días en curar.

La fiebre, el grave compromiso del estado general, la hematuria, la hemoglobinuria y la ictericia precoces marcan la gravidad del caso. Si aparecen antes de las 24 horas significan extrema gravedad. Si ninguno de estos síntomas y signos se presentan en las primeras 48 horas sólo habrá loxoscelismo cutáneo.

En el loxoscelismo visceral que va a la curación, las manifestaciones patológicas comiezan a atenuarse entre las 70 y las 80 horas. Ya dijimos que en nuestros 22 casos de loxoscelismo cutaneo-visceral tuvimos 7 muertos, es decir 31.81% de ellos.

Tratamiento — Tiene gran importancia que sea precoz. El ideal es usar suero específico. Desgraciadamente, entre nosotros se consigue difícilmente. Cuando se dispone de él debe emplearse en dosis suficiente y no atenerse a que en las instrucciones se dice que una ampolleta, por vía intramuscular es lo indicado, sino que poner dos, tres y muchas más si el caso lo exige. En el excelente suero que se prepara en el Instituto Butantan 5 ml. neutralizan 50 dosis mínimas necrosantes para la oreja de un conejo (15).

En su defecto, se han empleado desensibilizantes, antialérgicos, corticoides, analgésicos y tratamiento sintomático.

En los emponzoñamientos producidos por picaduras de arañas es preferible no emplear la vía digestiva porque el tubo digestivo se afecta y la absorción es mala.

No habiendo suero específico, y aún con él, lo más útil ha sido el empleo de corticosteroides, cuanto más luego mejor, en dosis altas, por vía parenteral, cortisona 800 milígramos al día los 4 primeros días y después 100 milígramos diarios hasta cuando sea necesario. El depomedrol (methyl prednisolone) 80 mg. intramusculares prontamente repitiendo cada 12, 24 o 48 horas según necesidad, disminuyendo despúes las dosis cada 24 horas (16, 17).

Hemos usado también toda la gama de antialérgicos y desensibilizantes, siempre de acción muy inferior a los corticoides.

La ligadura, la incisión, la cauterización, las inyecciones perifocales de permanganato de potasio, el amoníaco, etc., son ineficaces.

Como es natural, hay que usar también tratamiento sintomático, mas que nada para combatir el dolor, la sensación de quemadura, el insomnio y la angustia, así como estimulantes cardíacos y generales, sueros glucosados y transfusiones cuando la situación lo requiera.

El tratamiento local deberá ser cuidadoso y vigilante. Si la úlcera es muy grande y la cicatrización tórpida habrá que recurrir a injertos de piel.

Summary

Loxoscelism is clinically studied through 200 cases in Chile. The venom of *Loxosceles laeta* acts on the skin, determining a cutaneous form or "Grangenous spot", or on the skin and visceras, causing a very serious poisoning with a high mortality. Clinical differences between the two forms are described. In the visceral form, death occurs in 31.81% of these cases, 30 to 48 hours after the spider's bite. There is toxic hemolytic anemia, hepatic focal necrosis and hemoglobinuric nephrosis, with a series of tissue and general organic lesions. The differential diagnosis, prognosis and the results of treatment with specific sera and other medicaments are analysed.

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33(3):699-700, 1966

6. EL ARANEÍSMO EN BOLIVIA

J. VELLARD

Bolivia

Existen muy pocos datos concretos sobre el araneísmo en Bolivia.

La más antigua es del año 1886 (E. Simon, Ann. Soc. Ent. Belgique).

Un químico establecido en Cochabamba, el Dr. Sacc, envió a E. Simon diversas arañas designadas como *mico-mico*, sindicadas de producir accidentes graves: la picadura es igual al dolor producido por un hierro al rojo, seguido de violenta inflamación; la sangre pasa en las orinas y la muerte puede sobrevenir en algunas horas. Las especies recibidas por E. Simon fueron determinadas como *Dendryphantes* y *D. sacci*, de la familia de las SALTICIDAE, pero la atribución de accidentes a estas arañas carecía totalmente de bases. El *mico-mico* es en realidad el *Latrodectus mactans*.

Cuatro regiones biogeográficas principales deben ser consideradas para Bolivia:

En las altas regiones andinas, arriba de los 3.000 m, no existen arañas peligrosas. El veneno de las mayores especies del Altiplano *Lycosa rufimanoides* y *L. guaquiensis* es muy poco activo para el hombre.

En los valles secos, al oriente de los Andes, entre 1.500 y 2.800 m, principalmente, se observan dos formas de araneísmo: un araneísmo del campo producido por *Latrodectus mactans*, el mico colorado; y un araneísmo domiciliario cuyo responsable es un *Loxosceles* que no he podido determinar por haber recibido únicamente hembras.

No existe ningún dato estadístico al respecto, pero los médicos de Cochabamba y de Sucre conocen bien estos accidentes, que a veces son mortales.

La sintomatología no difiere de la observada en otros paises.

Solo notaré después de la picadura de *L. mactans*, un pequeño edema local duro alrededor del punto de inoculación del veneno. Los otros síntomas son clásicos: dolor irradiante violento, cefáleas, raquialgia, dolores abdominales, contracturas musculares. espasmo de la musculatura lisa. El restablecimiento es la regla, pero se ha observado algunos casos mortales a 2 o 3 días, por anuria.

Durante muchos años todos los casos de araneísmo de estas regiones fueron atribuidos a *L. mactans*.

Con la colaboración de diversos clínicos de Cochabamba, entre ellos el Dr. Hermógenes Sejas, he podido identificar a otra especie, la *araña negra*, un *Loxosceles sp*, que produce los accidentes más graves, con frecuencia mortales, con el cuadro habitual de loxoscelismo, edema, flictenas, necrosis local, ictericia, hematuria.

En la región del Chaco las THERAPHOSIDAE conocidas localmente por su nombre guaraní de ñandú-guassú son muy temidas, así como la añarimba, *Phoneutria rufibarbis*, pero no se han publicado relaciones clínicas de accidentes. En la región tropical de tipo amazónico, del Oriente Boliviano faltan también datos positivos. El pueblo teme diversas especies de araña; las THERAPHOSIDAE, los grandes Ctenídeos son frecuentes. Es muy probable que los *Phormictopus*, y *Acanthoscurria* sean responsables de accidentes. Es un campo de investigaciones todavia abierto.

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7. ULTRAESTRUTURA DO APARELHO VENENÍFERO DE *APIS* (*HYMENOPTERA, APIDAE*)

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Introdução

Os acessórios do ferrão são conhecidos nas abelhas com detalhes, desde o trabalho de Bordas(1), que fêz um estudo comparativo dêste órgão em várias espécies de himenópteros. Em *Apis*, como nas demais abelhas providas de ferrão, existem duas glândulas associadas à base dêsse órgão. A principal delas consiste de túbulo longo, fino e convoluto, localizado na parte posterior do abdomen. O túbulo termina apicalmente por uma bifurcação sob a forma de duas pequenas dilatações secretoras. Posteriormente, o túbulo se abre num reservatório, o saco de veneno (2). Esta glândula denomina-se ácida devido ao pH de sua secreção, e é aquela que produz o veneno.

A segunda glândula não se acha ligada ao ferrão, de maneira que a sua secreção possa ser injetada durante a picada, tendo sido denominada glândula básica.

Kerr e Lello (3) sugeriram a possibilidade da glândula ácida em *Bombus* ser secretora em tôda a sua extensão, estando porém a atividade mais intensa restrita à parte apical bifurcada. Sugeriram também que o saco de veneno poderia exercer função secretora. Por outro lado, Autrum e Kneitz (4), verificaram em *Apis*, que a secreção do veneno iniciava-se no final da pupação, pouco antes da emergência, atingindo seu máximo entre 10 a 16 dias de vida da abelha adulta.

No presente trabalho, pretende-se descrever a morfologia e ultraestrutura da parte apical bifurcada (glândula pròpriamente dita), e mediana da glândula ácida e do saco de veneno de pupas prestes a emergir e de abelhas adultas, campeiras.

Material e métodos

a. *Abelhas utilizadas* — 1. Pupas prestes a emergir, de operárias de abelhas híbridas (*Apis mellifera ligustica* e *A. mellifera adansonii*); 2. Operárias campeiras (aproximadamente 30 dias de idade) dos mesmos híbridos.

Pesquisa realizada sob os auspícios da Fundação de Amparo à Pesquisa do Estado de São Paulo, Conselho Nacional de Pesquisas e Organização dos Estados Americanos.

b. *Técnicas histológicas para microscopia eletrônica* — As abelhas foram dissecadas em solução fisiológica (NaCl a 0,7%) tamponada para pH 7,1 com tampão fosfato, tendo sido estas abelhas prèviamente anestesiadas com resfriamento a 0°C durante 15 minutos. As glândulas e o saco de veneno, após sua extração, foram fixados em solução de tetróxido de ósmio (5) ou numa combinação de tetróxido de ósmio e permanganato de potássio (6). A desidratação foi efetuada em acetona e o material fixado, foi incluído em Epon 812 (7). Após serem seccionadas em ultramicrótomo Porter Blum, MT-2, as secções ultrafinas foram contrastadas em acetato de uranila, durante 1 hora, e a seguir em hidróxido de chumbo (8), e examinadas em microscópio eletrônico Elmiskop I da Siemens.

Resultados

A. *Glândula ácida* — A fim de facilitar a exposição dos resultados, esta glândula foi dividida em duas porções arbitrárias, respectivamente, a parte apical, bifurcada e a porção mediana. Não se estudou a porção basal, em contacto com o saco de veneno, pois esta não difere histològicamente da região mediana.

1. *Pupas* — Nas pupas, a luz da glândula ácida, na extremidade apical dêste órgão, tem secção de forma estrelada e sua parede é formada de uma espêssa cutícula (ca. 5 μ), de material provàvelmente não esclerotizado, disposto em camadas concêntricas.Em tôrno desta cutícula achavam-se dispostas as células secretoras. Estas apresentavam-se com grandes vacúolos, dando à parte secretora um aspecto rendilhado. Não se observou uma membrana conspícua, limitando tais vacúolos (Fig. 1a). A semelhança do que ocorre em células vegetais, devido à presença dos enormes vacúolos, os núcleos destas células encontravam-se em geral comprimidos em pequenas faixas do citoplasma.

A porção citoplasmática destas células era rica em ribossomas, embora pobre em elementos do retículo endoplasmático (Fig. 1). Nas junções celulares, as membranas justapostas se apresentavam sinuosas nas secções, e em alguns pontos formavam alças. O espaço entre estas membranas justapostas achava-se preenchido por um substância densa, e em certas regiões elas se espessavam, o que talvez pudesse representar desmossomas, muito embora não tivessem um aspecto característico destas estruturas celulares. Nesta fase, os mitocôndrios ocorriam em pequeno número, uniformemente distribuídos nas células e se caracterizavam por abundantes cristas. Zonas de Golgi foram raramente observadas e em nenhum caso pôde-se identificar as vesículas de secreção, usualmente a elas associadas.

Dois tipos de núcleos foram constatados nestas células. Um grande, aproximadamente esférico, que representaria o núcleo da célula secretora de localização mais periférica e outro, menor, localizado próximo aos canalículos intracelulares ou à cutícula que reveste a luz da glândula. Nos núcleos maiores, notavam-se vários nucléolos bem desenvolvidos e um retículo de material cromático (Fig. 1). Disperso no nucleoplasma, apresentava-se grande número de grânulos densos, semelhantes aos ribossomas. Os núcleos pequenos não apresentavam nenhuma característica marcante. Aparentemente, os núcleos pequenos e os maiores estavam contidos nas mesmas células, isto é, nesta fase não se percebiam membranas divisórias compartimentando o citoplasma pertencente a cada tipo de núcleo. As células desta porção, como as da parte mediana e as do saco de veneno, apresentavam uma série de canalículos intracelulares. Tais canalículos

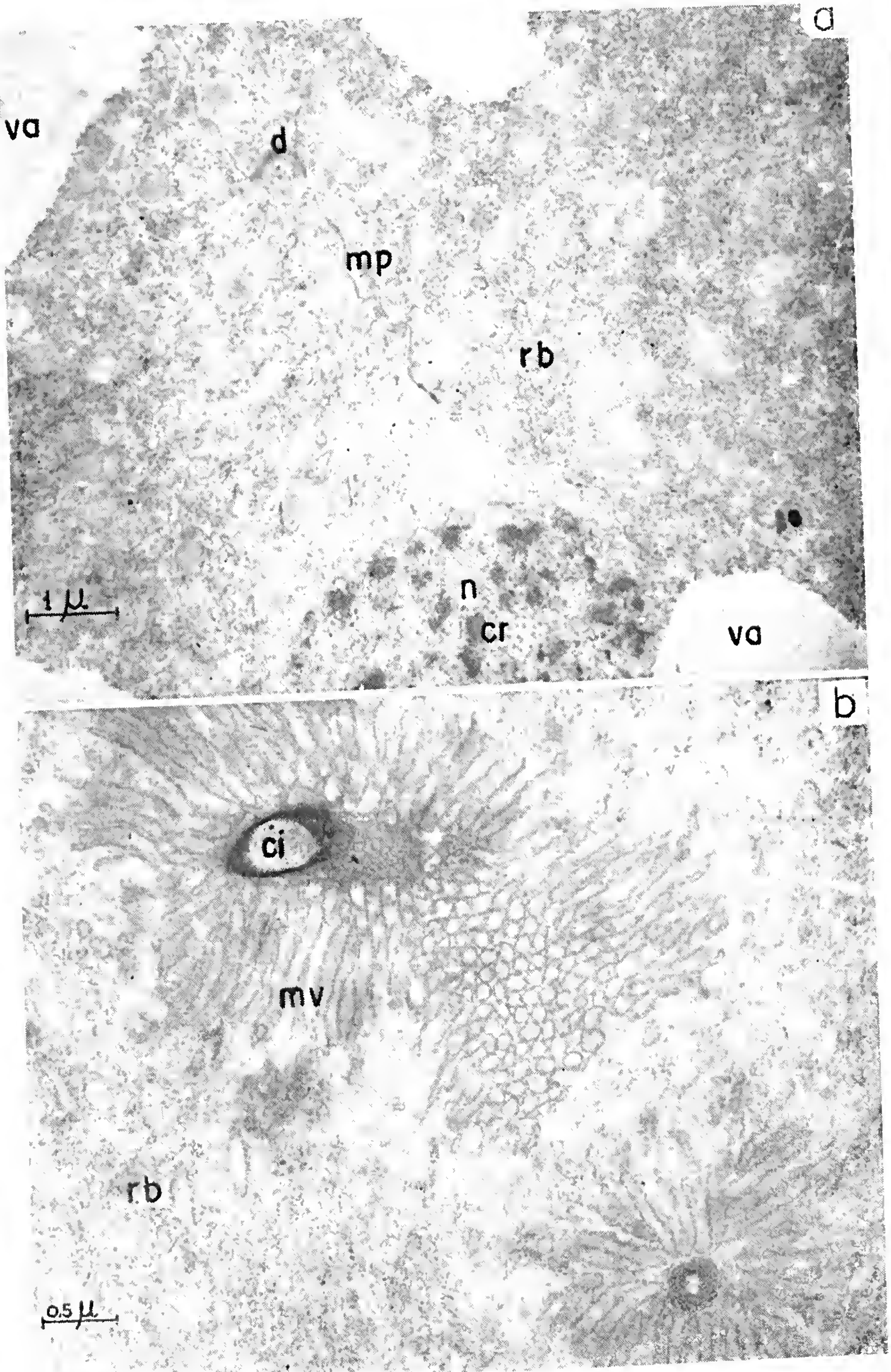

Fig. 1a – Micrografias da porção apical da glândula ácida da pupa, vendo-se grandes vacúolos (Va) sem membrana limitante, um retículo pouco desenvolvido (r) mas numerosos ribossomas (rb). As membranas separatórias das células (mp) têm espessamentos que podem ser tomados como desmossomas (d). Os canalículos intercelulares (ci) apresentam microvilosidades (mv) ao seu redor. Material fixado em Palade.

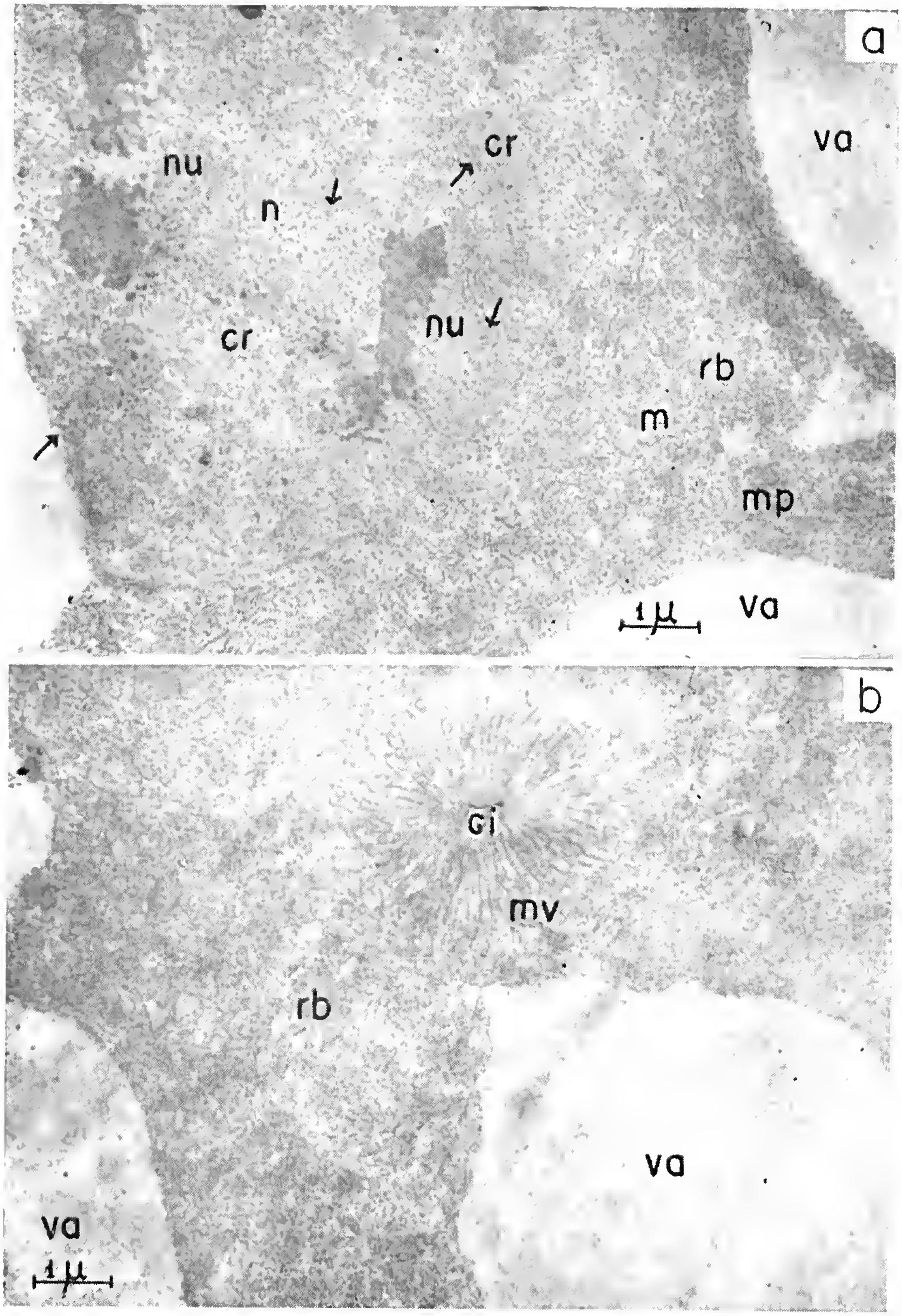

Fig. 1 — Micrografias da porção apical da glândula ácida de pupa, vendo-se os grandes vacúolos (Va) cheios com material pouco denso, os núcleos (n) comprimidos no citoplasma entre os vacúolos, canalículos intercelulares (ci) com microvilosidades (mv) ao seu redor, numerosos ribossomas (rb) e poucas lamelas do retículo (r). Material fixado em Palade.

possuíam uma parede densa, possìvelmente de quitina esclerotizada, disposta em camadas concêntricas e em sua luz ocorria uma substância granular de densidade média. Dois tipos de canalículos puderam ser distinguidos: um de diâmetro maior e de paredes mais espêssas, localizado preferencialmente próximo à luz da glândula (Fig. 2a); e outro mais fino, de paredes mais delgadas. Êstes últimos possìvelmente representavam ramificações menores dos primeiros e se caracterizavam por serem de menor calibre e se acharem envolvidos por microvilosidades da célula (Fig. 1 e Fig. 2a). Estas microvilosidades mediam cêrca de 1 μ de comprimento e 20-25 mμ de diâmetro e entre elas não se pôde notar nenhum material denso.

A região mediana da glândula ácida da pupa apresentava um aspecto muito semelhante ao descrito para a zona apical. As únicas diferenças encontradas foram quanto à presença de pequenas vesículas de cêrca de 30 mμ de diâmetro, contendo material relativamente denso, aparentemente associadas às microvilosidades em tôrno dos canalículos intracelulares. Os vacúolos eram bem menores do que os da região apical.

2. *Abelhas campeiras* — A porção apical da glândula dêstes indivíduos apresentava-se semelhante à da pupa, mas diferenciava-se pelo fato do citoplasma apresentar-se extremamente vesiculado e pela ausência dos vacúolos (Fig. 2a). Tais vesículas provàvelmente representariam elementos do retículo endoplasmático agranular alargados, mas com um conteúdo de baixa densidade.A zona citoplasmática, adjacente às microvilosidades apresentava-se menos densa e livre de orgânulos celulares, talvez refletindo um acúmulo de secreção, não organizada nesta região (Fig. 2a). Freqüentemente observou-se um alargamento do espaço entre as microvilosidades adjacentes, na porção basal, formando-se pequenas bôlsas. Todavia, a diferença mais marcante, quando comparada esta região das abelhas adultas com a das pupas, era a ausência quase total dos enormes vacúolos, ocorrendo êstes, em tamanho e número bem reduzido e próximos à luz da glândula, condição semelhante à verificada na região mediana da glândula da pupa. Nesta fase é possível perceber uma membrana plásmica divisória, próxima aos canalículos maiores.

Em abelhas campeiras, as células da região mediana da glândula apresentavam-se com sinais evidentes de degeneração, pràticamente sem orgânulos celulares distintos, restando sòmente identificáveis o núcleo e as vilosidades em tôrno dos canalículos. O resto do espaço intracelular achava-se preenchido por vesículas de forma e dimensões irregulares. Estruturas em forma de lamelas concêntricas aparecem freqüentemente nestas células e provàvelmente correspondem aos parassomas (p), que surgem caracterìsticamente em células em vias de degeneração (Fig. 2b).

B. *Saco de veneno* — Distinguem-se duas regiões no saco de veneno: a porção apical, onde desemboca a glândula ácida, formada de células relativamente altas e a zona basal, próxima ao ducto excretor, constituída de células bem baixas. A cutícula, revestindo êste saco, era muito espêssa, principalmente na parte basal, onde mostrava organização típica do exoesqueleto de insetos. Na parte apical, esta cutícula assemelhava-se à da glândula ácida. As células do saco de veneno ostentavam também canalículos intracelulares e pequenos núcleos próximos à cutícula e aos canalículos.

Na pupa, as células do saco de veneno diferenciavam-se das da glândula ácida por apresentarem invaginações da membrana plásmica associadas a mitocôndrios, na zona cortical. Nesta célula, pequenos bastonetes ou grânulos densos,

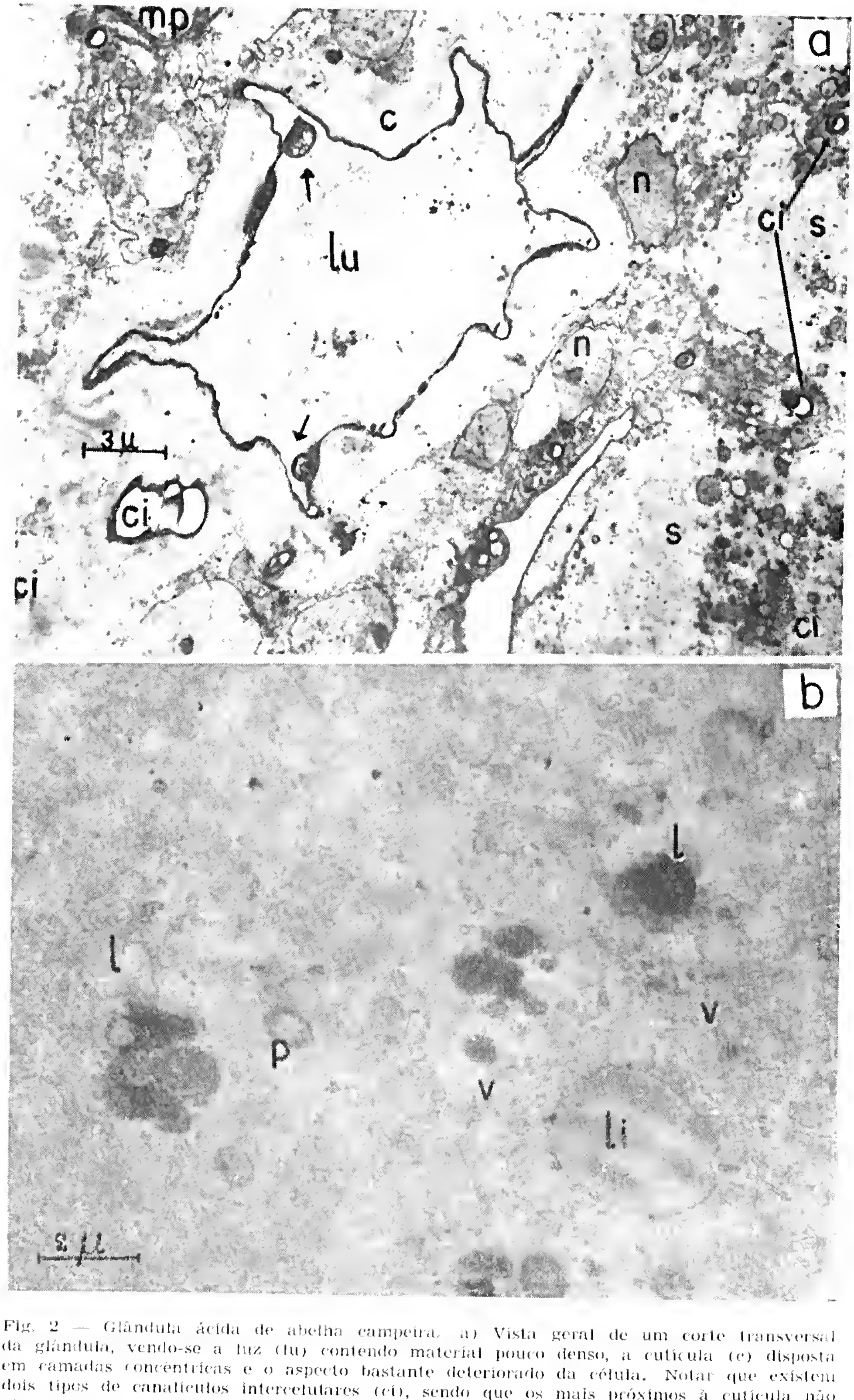

Fig. 2 — Glândula ácida de abelha campeira. a) Vista geral de um corte transversal da glândula, vendo-se a luz (lu) contendo material pouco denso, a cutícula (c) disposta em camadas concêntricas e o aspecto bastante deteriorado da célula. Notar que existem dois tipos de canalículos intercelulares (ci), sendo que os mais próximos à cutícula não têm microvilosidades ao redor. Os pontos marcados com s correspondem a depósitos de secreção. b) Detalhe da célula mostrando estruturas parecidas com os lisossomas (l) e estruturas lamelares concêntricas (p).

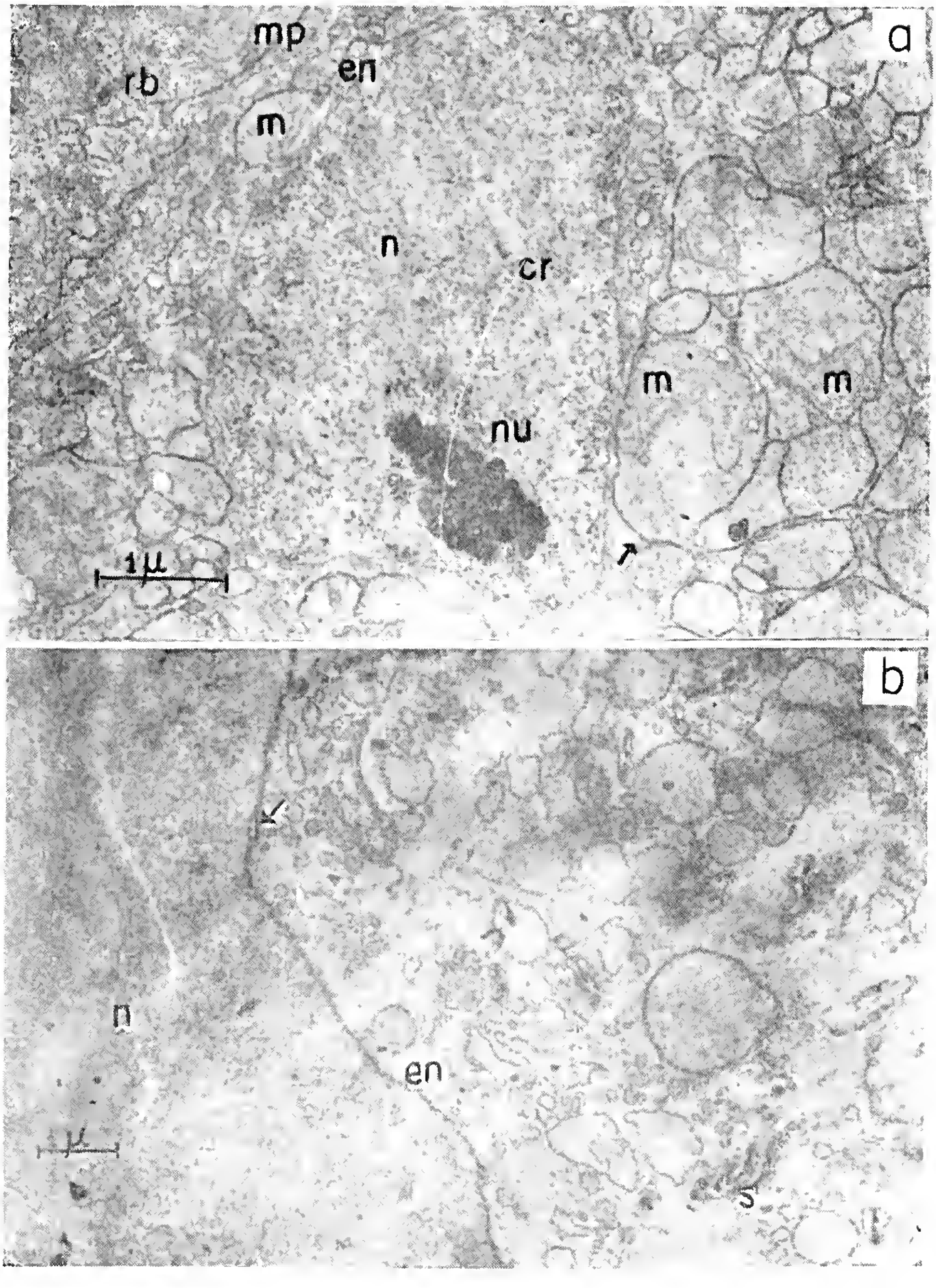

Fig. 3 — Saco de veneno. a) Vesículas contendo mitocôndrios em degeneração no interior. b) Origem de secreção a partir do núcleo. Bastões densos (bd) vêem-se primeiro entre as membranas do envoltório nuclear (en) e depois no interior de vesículas (v) no citoplasma.

de dimensões variadas (75-100 mμ $\times$ 100-400 mμ), foram encontrados (Fig. 3b). Êstes ocorriam livres no citoplasma, agrupados ou individualmente no interior de vesículas e também freqüentemente no espaço perinuclear (Fig. 3b). Mitocôndrios, de forma atípica, foram encontrados em células de abelhas adultas. Êstes mitocôndrios apresentavam-se com poucas cristas, e um substrato muito denso e embora alguns ocorressem normalmente no citoplasma, outros apareciam freqüentemente em diversas fases de degeneração, encerrados em vesículas (Fig. 3a). Além destas vesículas contendo mitocôndrios, numerosas outras, contendo um material de baixa densidade ocorrem nestas células. Tais vesículas parecem aumentar à medida que envelhece a abelha e, presumìvelmente, originam-se do retículo endoplasmático. Na base das microvilosidades que circundam os canalículos intracelulares, freqüentemente observam-se pequenas e numerosas vesículas e também gotículas densas, provàvelmente de natureza lipídica. As paredes dos canalículos, por outro lado, ocasionalmente mostravam interrupções semelhantes àquelas descritas na glândula hipofaríngea de *Apis* (9).

Estruturas consideradas como lisossomas foram freqüentemente encontradas nestas células do saco de veneno. Tais orgânulos eram limitados por uma membrana simples e continham grânulos densos e lamelas concêntricas em seu interior. Nas células da região mediana do saco de veneno foram encontradas diferentes fases de evolução destas estruturas.

A musculatura que envolve o saco de veneno, bastante desenvolvida, dispunha-se tanto circular como longitudinalmente. As fibras desta musculatura eram do tipo estriado, embora não apresentassem tôdas as características de uma musculatura estriada típica. As células musculares eram alongadas tendo miofibrilas apenas na porção central. Tais fibrilas mostravam, a espaços proximados de 3 μ, linhas densas que corresponderiam à linha Z (10). Lateralmente às miofibrilas, elementos do retículo endoplasmático e mitocôndrios foram observados. Tal tipo de musculatura é encontrado comumente em vísceras de insetos.

Traquéias, com armação helicoidal de quitina, foram freqüentemente encontradas nas células da glândula ácida e do saco de veneno. Membrana basal, espêssa, de densidade mediana estava invariàvelmente presente nas células glandulares e do saco de veneno, bem como nas musculares.

Discussão

Tôda a glândula ácida, da pupa de *Apis*, parece ter atividade secretora, confirmando e até reforçando as observações de Autrum e Kneitz (4). Porém, em abelhas de 30 ou mais dias de idade, a glândula ácida acha-se degenerada, restando parcialmente intacta apenas a porção apical. Estas observações sugerem que o processo degenerativo destas glândulas progride da parte basal para a apical. O veneno produzido pela abelha durante a fase final da pupação e primeiros dias da vida adulta é armazenado no saco de veneno até o momento do uso. Geralmente as operárias de *Apis* picam uma vez só porque, ao fazê-lo, perdem o ferrão, essa talvez seja a causa da degeneração precoce da glândula ácida. Em *Bombus* (3) mesmo nas abelhas campeiras, tôda a glândula é secretora, mas esta abelha não deixa o ferrão ao picar. Fenômeno similar ocorre no saco de veneno, onde principalmente as células apicais passam por processos degenerativos.

Particularmente notável é a ausência pràticamente total do complexo de Golgi, neste sistema venenífero, pois sua importância nos fenômenos de secreção

é bastante conhecida (5).Esta ausência talvez seja explicável pelo fato de a secreção ser armazenada, intracelularmente, em enormes vacúolos na fase pupal, pois o complexo de Golgi teria mais função como concentrador da secreção.

Nas abelhas adultas, aparentemente haveria a secreção de substâncias diferentes daquelas produzidas durante a pupação e eliminadas nos primeiros dias da vida adulta.

Os bastonetes ou grânulos no saco de veneno, que migrariam do espaço perinuclear para o citoplasma, poderiam representar uma destas substâncias. O envolvimento do núcleo em atividades secretoras já foi relatado em hipotálamo de rato associado à neurosecreção (11). Também as vesículas associadas ou não a mitocôndrios, poderiam conter outro tipo de secreção, embora mais pareçam um resultado de degeneração celular.

O conteúdo dos enormes vacúolos encontrados em células das glândulas ácidas de pupa representaria uma secreção ainda não eliminada. Possìvelmente nas fases subseqüentes da existência da abelha, tal secreção seria lançada para a luz da glândula, sendo armazenada aí ou no saco de veneno, pois em células de abelhas adultas, em vias de degeneração, não há sinal de acúmulo desta secreção.

É provável que a glândula ácida produza uma parte do veneno, ao qual são adicionadas posteriormente outras substâncias, secretadas pelo saco de veneno. Estas últimas, poderiam determinar a especificidade do veneno de cada espécie de abelha, uma vez que se originam em íntima relação com o núcleo.

Evidentemente os canalículos intracelulares devem funcionar como um sistema coletor da secreção. Tais canalículos facilitariam a eliminação da secreção aumentando a superfície polarizada (12, 9). As dobras da superfície celular em forma de microvilosidades que envolvem os canalículos, principalmente nas suas ramificações menores, proporcionariam uma superfície maior para a eliminação da secreção. É provável ainda que, principalmente na glândula ácida, em que a secreção parece ser mais fluida, parte do solvente possa ser reabsorvido pelas microvilosidades antes da secreção passar para a luz do canalículo, o que representaria uma economia de água para a célula.

A origem dos canalículos intracelulares parece ser diferente da das células secretoras. Freqüentemente, adjacentes à cutícula e aos canalículos, estão células achatadas e de núcleo pequeno. As membranas separatórias entre estas células e as secretoras não são visíveis nas abelhas jovens, mas tornam-se evidentes nas abelhas campeiras. Isto reforça a teoria (4) da origem independente das células secretoras e formadoras da cutícula e canalículo. Neste caso a cutícula seria uma verdadeira íntima.

As presentes observações confirmam também o ponto de vista de que os canalículos intercelulares estão, na realidade, topològicamente fora das células secretoras e portanto sua denominação é incorreta (12). Tais canalículos apresentam-se tìpicamente separados da célula secretora por uma membrana plásmica, normal ou dobrada em forma de microvilosidades.

O aparecimento de estruturas consideradas como parassomas e lisossomas, além da extensa vacuolização do citoplasma, anomalias no núcleo, etc., devem representar inegàvelmente, sinais flagrantes da degeneração das células glandulares e do saco de veneno nos indivíduos adultos.

A musculatura em volta do saco de veneno deve estar associada à injeção do veneno na vítima, durante o ato da picada.

Summary

Electron microscope studies were made on acid gland and venom sac of *Apis* workers. Workers of *Apis* are studied in the pupal phase and adult foragers (30 days older). In pupa all lenght of acid gland and the apical portion of venom sac was secretory and probably was in great activity. In adult workers the cells of acid glands and the venom sac degenerate. The degeneration was less intense in the apical portion of the acid gland.

In pupa acid gland cells, appear enormous vacuoles that possibly contain the secretion of this phase. In adults this vacuoles practically desappear. It was suggest that the adults secrete another type of substances. In the venom sac dense material in form of rods or small granules free or within vesicles seem to be the secretion. These materials appear in the cell always associated with the nucleus, first located in the perinuclear space.

The venom sac is provided with a powerful striated musculature, that probably add in the venom injection.

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8. DONNÉES HISTOCHIMIQUES SUR LA GLANDE À VENIN (GLANDE CHÉLICÉRIENNE) DES ARAIGNÉES DIPNEUMONES

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"That such minute glands should secrete enough venom to kill human beings... is hard to believe" (1).

"Une piqûre dont ma loupe ne peut trouver les traces... a suffi... pour tuer la vigoureuse bête. Toute proportion gardée, le Crotale, le Céraste, le Trigonocephale, et autres serpents d'odieux renom, n'obtiennent pas, sur leurs victimes, des effects aussi foudroyants" (2).

C'est à une telle estimation qu'était conduit le célèbre entomologiste en observant l'effet du venin des Epeires, fasciées ou soyeuses, sur la grande libèllule *Aeschua grandis* L. et un observateur contemporain a pu dire, sans exagération semble-t-il, que les araignées venimeuses (en particulier, *Latrodectus* et *Loxosceles*) tuent autant d'humains que les guêpes, aussi, leur petite taille, comparée à celle de l'être humain, en fait-elle "de grands tueurs". Il est donc parfaitement étonnant qu'une sécrétion aussi puissamment dévastatrice n'ait pas encore fait l'objet de recherches comparées méthodiques, quant à sa nature réelle et aux particularités de la structure des cellules qui l'élaborent, chez des représentants des divers genres d'Arachnides. Les quelques données dont nous disposons sont si rudimentaires qu'en 1964, encore, Bücherl admettait que de nouvelles recherches étaient nécessaires.

Cependant, des travaux récents ont précisé la nature, le plus souvent fondamentalement protidique du venin des araignées, ainsi que l'existence d'importantes variations spécifiques; s'est ainsi que le venin de *Phoneutria fera* est cinq fois moins riche en lysine et trois fois moins riche en histamine que le venin de *Lycosa erythrognatha* (3). Le venin sec de cette lycose est remarquablement riche en histamine quand on le compare à celui de *Phoneutria fera*; il contient, en effect, de 14 à 19 mg/g d'histamine quand celui de *Phoneutria* n'en contient que de 0,6 à 0,8 mg/g (4). Par contre, le venin de *Phoneutria* est environ deux fois plus riche en glutamine (3) que celui de *Lycosa*; c'est grâce à cette nature essentiellement protidique qu'il est possible de préparer des antivenins efficaces.

Nous savons aussi que le venin de certaines araignées est riche en sérotonine et en polypeptide de type bradykinine; le venin de *Lycosa* contient environ trois fois plus de sérotonine que celui de *Phoneutria* (soit 1,5 à

1.9 mg/g); par contre, il est infiniment plus pauvre en polypeptide que celui de *Phoneutria*, quand il a une activité de 1.5 à 5 unités de bradykinine, le venin sec de *Lycosa* a une activité de 360 à 420 unités de bradykinine (4).

Cependant, la plus large part du venin de certaines araignées n'est pas protidique; chez *Atrax robustus*, par exemple, le venin est décomposable, par électrophorèse, en trois fractions toxiques, de mobilités bien différentes, dont l'une est dépourvue d'acides aminés; c'est à cette particularité que Wiener (5) attribue l'impossibilité d'obtenir un antivenin vraiment efficace contre les morsures de cette araignée.

Lebez (6) a trouvé des lipides dans le venin de *Latrodectus tredecimguttatus*; cependant, étudiant la même espèce, Cantore et Bettini (7) n'ont pas trouvé de lipides soudanophiles. A cet égard, il convient de rappeler que certains histologistes (8, 9) ont observé des inclusions osmiophiles dans les cellules glandulaires et le venin des ARACHNIDES; ils ont attribué cette osmiophilie, sans autre preuve d'ailleurs, à des lipides.

Fischer et Bohn (3) ont trouvé un pentose dans le venin de *Phoneutria fera* et de *Lycosa erythrognatha*; Gabe (10) a mis en évidence une glycoprotéine histochimiquement décelable dans le venin de 9 espèces d'Aranéides (appartenant à 7 genres) et Wiener (5) a décelé un glucide dans le venin d'*Atrax robustus*.

Nos connaissances sur les enzymes des venins des Araignées sont très succintes. Dès la fin du siècle dernier, Gaubert (11) admettait que la sécrétion des glandes venimeuses "doit renfermer des ferments qui modifient les liquides que l'animal absorbe par succion (p. 56); cependant, la plupart des auteurs n'ont envisagé pour le venin qu'un rôle de défense ou d'attaque, utile pour supprimer un adversaire ou pour immobiliser les proies. Comme le suggérait Millot (12) pour lever tout dout, quant au rôle du venin dans l'alimentation, "il faudrait rechercher *in vitro*, l'action digestive d'extraits des glandes", ce qui à ma connaissance n'a pas encore été fait. Quoi qu'il en soit sur ce point, Duran-Reynals (13) a vu que les extraits d'araignées contenaient un remarquable "facteur de diffusion" absent des èxtraits de sauterelle, de libéllule ou de fourmi... et ce facteur a été identifié depuis à l'hyaluronidase. En outre, le venin de la plupart des araignées qui ont été examinées à ce point de vue, a des effects protéolytiques et anti-acétylcholinestérasique (7).

Il semble que nos connaissances doivent désormais évoluer rapidement; en effect, l'électrophorèse, en particulier, est d'une aide précieuse; elle fait parfaitement apparaître la complexité du venin des araignées; suivant l'éspèce étudiée, suivant la technique d'électrophorèse mise en oeuvre, six fractions (14), cinq fractions (15, 16, 7, 17, 18), voire huit fractions (19) ont pu être dissociées.

Ainsi, à l'heure actuelle, nous sommes relativement mieux documentés sur les caractères du venin des araignées que sur la morphologie des glandes qui l'élaborent. Les mémoires classiques, dont un relevé soigneux figure dans le travail de Millot (12) ont, certes, abouti à des précisions intéressantes sur le développement embryonnaire de ces organes et leur anatomie chez l'adulte, mais les histologistes semblent s'être contentés d'appliquer les seules méthodes de l'anatomie microscopique, d'où une carence évidente de nos informations relatives aux caractères cytologiques et histochimiques des cellules glandulaires, ainsi qu'à leur mode de sécrétion. Parmi les articles de Traité portant sur des ARANÉIDES,

celui de Gerhardt et Kästner (20) reprend les notions essentielles du travail de Millot (12), sans leur ajouter d'élément nouveau. Millot (21), à son tour, a exposé, sous une forme plus concise, les mêmes données. Les quelques publications orientées vers la morphologie parues depuis cette date ne représentent pas un progrès, si bien que la parcimonie des documents disponibles, apparaît clairement à la lecture de la monographie de Savory (22). Il devait donc paraître opportun d'appliquer quelques techniques cytologiques et surtout histochimiques à ces glandes à venin, puisque seuls les composants glucidiques semblent avoir été explorés avec les techniques histochimiques récentes (10).

Materiel et techniques

Les caractères morphologiques généraux et certaines particularités cytologiques des glandes à venin ont pu être étudiées chez les espèces suivantes:

Dysdera crocata C. Koch.
Pholcus phalangioides Füssly.
Teutana grossa C. Koch.
Araneus diadematus Clerck.
Clubiona terrestris Westr.
Tegenaria parietina Fourcroy.
Tegenaria derhami Scopoli.

Le plus souvent le matériel a été fixé par le liquide de Bouin, inclus à la paraffine et débité en coupes sériées de 5 μ. Parmi les méthodes histologiques générales, l'azan de Heidenhain, le trichrome en un temps sans différenciation, le trichrome de Masson-Goldner et la coloration de Mann-Dominici nous ont fourni les images les plus significatives. Les variations anatomiques des glandes sont importantes d'un genre à l'autre et cependant aucune de ces techniques n'a fait apparaître de différences dans la structure histologique chez les sept espèces examinées (Tableau I).

TABLEAU I — AFFINITÉS TINCTORIALES DES PRODUITS DE SÉCRETION DE LA GLANDE CHÉLICÉRIENNE

Colorations	*Flaques*	*Grains*
Trichrome en un temps	vertes	rouges
Trichrome de Masson-Goldner	vertes	rouges
Azan	bleues	rouges
Mann-Dominici sans oxydation	roses	rouges
Mann-Dominici avec oxydation	roses	bleues
Mann biacide	bleues	rouges

La plupart des constatations histochimiques ont été obtenues sur des coupes de glande chélicérienne de *Tegenaria derhami* et de *Tegenaria parietina*; elles sont résumées dans le Tableau II.

TABLEAU II — CARACTÈRES HISTOCHIMIQUES DES PRODUITS DE SÉCRÉTION DE LA GLANDE CHÉLICÉRIENNE

Méthode	*Flaques*	*Grains*
APS	+	+
APS après diastase du malt	+	+
Réaction métachromatique	0	0
Coloration par la fuchsine-paraldéhyde	0	0
idem, après oxydation permanganique	+	+
Coloration au bleu alcian	0	0
Alloxane-Schiff	±	+
Tétrazoréaction de Danielli	±	+
Réaction au ferricyanure ferrique	+	0
idem, après blocage au sublimé	0	0
Azoréaction	0	0
Réaction argentaffine	0	0
Réaction du rosindole	0	+

RESULTATS

LA GLANDE

Rappel anatomique — Les glandes chélicériennes des Aranéides dipneumones sont piriformes, situées dans la région proximale et dorsale du prosoma. La partie renflée de ces glandes, sacciforme et plus ou moins longue, occupe un emplacement superficiel, très proche de la paroi dorsale du corps (Fig. 1 à 3); sa face inférieure est plus ou moins adjacente, suivant les espèces, aux diverticules thoracentériques et à la masse nerveuse sus-oesophagienne; les parois latérales des glandes sont au contact des muscles du prosoma. La partie antérieure effilée de la glande se continue par un canal qui parcourt toute l'étendue de la chélicère, pour déboucher dans l'article terminal de cette pièce, non loin de l'extrémité du crochet. L'exploration d'un matériel abondant (23, 24, 25, 26, 9, 27, 28, 12, 29, 1, 8, 30), a révélé des différences de taille énormes suivant les genres; c'est ainsi que la glande chélicérienne est petite chez *Amaurobius erberi*, moyenne chez *Helecnemus pluchei* et grande chez *Scytodes delicatula* (27); elle est entièrement contenue dans la chélicère, chez la *Mygale*, elle s'étend dans l'article proximal de la chélicère chez *Clubiona pallicula* et elle est entièrement dans le céphalothorax chez *Epeira*, *Agelena* et *Tegenaria* (23); elle est relativement énorme chez les Pholcides et la Filistate (24). La taille de la glande est sans rapport avec la toxicité du venin, ou le comportement de l'araignée; les cas extrêmes sont représentés par la Filistate et les Uloborides; en effect, la Filistate, qui possède une énorme glande à venin, est apparemment incapable de mordre, car les deux chélicères sont soudées à leur base et sur une partie de leur étendue (il en est de même chez les Pholcides). Au contraire, chez les Uloborides, il n'existe pas de glande à venin, mais la chélicère est fonctionnelle et les crochets peuvent mordre.

La glande à venin est composée d'une musculeuse, d'une membrane basale et d'un épithélium sécréteur; le canal excréteur de la glande est dépourvu de

musculeuse; sa paroi est très mince. elle ne comporte qu'un basale tapissée d'un épithélium dont les caractères cytologiques diffèrent de ceux de la partie proprement glandulaire.

Caractères histologiques — La musculeuse qui engaîne le sac glandulaire est composée de fibres striées dont la structure est semblable à celle des muscles du prosoma, mais qui sont remarquables par leur disposition en spires régulières autour de la glande (Fig. 1 à 3).

Les auteurs classiques ne mentionnent pas l'existence d'une basale, sur laquelle repose l'épithélium glandulaire; Legendre (1935) signale une basale mince chez *Tegenaria*; en fait, cette basale est assez épaisse, homogène et pourvue des affinités tinctoriales habituelles aux basales glandulaires: elle est fortement cyanophile, elle retient le vert solide du trichrome en un temps, le vert lumière du trichrome de Masson-Goldner, le bleu d'aniline de l'azan. La réaction à l'acide periodique-Schiff lui confère l'habituelle teinte rouge intense. signalétique de composés glucidiques; elle ne contient apparemment pas de mucopolysaccharides acides.

L'épithélium glandulaire est fait de cellules prismatiques, très hautes et étroites, à parois minces et fragiles; le plus grand diamètre peut atteindre 100 μ, la largeur est voisine de 10 μ. Chez certaines espèces, il existe des inégalités de la hauteur de l'épithélium glandulaire; signalées dès 1880 par Mac Leod, chez *Epeira diademata*, elles ont été décrites par Bordas (26), chez *Latrodectus tredecimguttatus*; ces inégalités peuvent aller jusqu'à la diposition en éventail des cellules, et un véritable cloisonnement de la lumière glandulaire par des replis épithéliaux existe chez *Filistata insidiatrix* (12); une telle disposition des cellules glandulaires n'existe pas chez les espèces étudiées ici (Fig. 1 à 3).

Les noyaux des cellules glandulaires sont de petite taille et basaux; ils apparaissent régulièrement arrondis sur les coupes de la glande; ils mesurent en moyenne 8 μ de diamètre; leur chromatine apparaît en mottes irrégulières, uniformément réparties dans le cytoplasme; il existe un petit nucléole, fortement basophile. Les mitoses dans l'épithélium des glandes chélicériennes des araignées adultes sont extrêmement rares.

Le cytoplasme basal, périnucléaire, tranche sur le reste du corps cellulaire, dès l'examen des préparations colorées par les techniques histologiques générales (Fig. 4); il est, en effet, pourvu d'une forte affinité pour les colorants basiques et la disparition de cette basophilie après mise en oeuvre de la ribonucléase, démontre que la basophilie est due à des ribonucléines (Fig. 5).

La partie supré-nucléaire du cytoplasme constitue, en raison de la position très basale des noyaux, la majeure partie du corps cellulaire; son aspect varie avec les stades du cycle sécrétoire; dans certaines cellules, elle apparaît entièrement vidée (Fig. 8), alors que dans d'autres, elle contient une quantité plus ou moins considérable de sécrétion (Fig. 6 et 7).

Le produit de sécrétion de la glande chélicérienne n'est pas unique: la seule morphologie suffit pour distinguer deux substances. En effet, l'un des produits sécrétés forme des flaques plus ou moins étendues et des trainées, qui parfois remplissent toute la partie supra-nucléaire des cellules, tandis que l'autre se présente sous forme de granules et de gouttelettes, aux contours bien définis, dont la taille ne dépasse généralement pas 1,5 μ; ces deux produits coexistent souvent (Fig. 4, 5 et 9), dans une même cellule épithéliale.

Les affinités tinctoriales des deux produits de sécrétion sont fondamentalement différentes. En effet, la sécrétion en flaques est faiblement éosinophile après mise en oeuvre de la technique de Mann-Dominici et elle est cyonaphile avec les colorations trichromes utilisées ici; le produit retient fortement le vert lumière du trichrome de Masson-Goldner, le vert solide du trichrome en un temps de Gabe et Martoja, le bleu d'aniline de l'azan de Heidenhain. Au contraire, le produit de sécrétion en granules ou en gouttelettes est fortement éosinophile après coloration suivant Mann-Dominici, lorsque cette méthode est pratiquée sans oxydation préalable des coupes; après oxydation, il devient au contraire, fortement basophile; érythrophile avec les colorations trichromes (Fig. 4 et 5), ce produit retient la fuchsine acide, l'azorubine S et l'azocarmin (Tableau I).

Caractères histochimiques — Certains caractères histochimiques sont communs aux deux produits de sécrétion de la glande chélicérienne (Tableau II). C'est ainsi que les flaques aussi bien que grains sont APS-positifs et dépourvus de glycogène, aussi bien que de mucopolysaccharides acides; il semble que leur réactivité à l'acide périodique-Schiff traduise, soit la présence de muco polysaccharides neutres, soit de glycoprotéines, comme l'a admis Gabe (10).

Les colorations par l'acide periodique-Schiff et par la fuchsine paraldéhyde (Fig. 6 à 8) permettent d'apprécier parfaitement la plus ou moins grande abondance du produit de sécrétion. Certains caractères histochimiques révèlent la dualité de la sécrétion de la glande chélicérienne; c'est ainsi que la recherche des protides fait apparaître des différences nettes entre deux constituants du venin; en effet, les flaques cyanophiles réagissent faiblement à l'alloxane-Schiff et à la tétrazoréaction de Danielli, alors que les grains érythrophiles réagissent très fortement. Inversement, la recherche des protides sulphydrilés fait apparaître nettement les flaques cyanophiles, alors que les grains restent inapparents et ne se colorent que par le colorant de fond, après mise en oeuvre de la technique au ferricyanure ferrique. La recherche de dérivés indoliques n'est positive que dans les grains. La recherche des polyphenols est restée totalement négative (Fig. 9 à 12).

Les différences histochimiques notées au niveau des cellules épithéliales du sac glandulaire, restent les mêmes dans la lumière du sac, où s'accumulent les deux produits après leur extrusion, si bien que leur distinction reste, là, aussi facile qu'au sein des cellules; elles persistent encore dans la sécrétion du canal excréteur.

LE CANAL EXCRÉTEUR

La limite entre le sac glandulaire et le canal excréteur est indiquée par des modifications anatomiques très nettes; d'une part, la lumière se rétrécit et d'autre part la tunique musculeuse de la glande disparaît. En outre, la hauteur des cellules épithéliales diminue brusquement pour n'être plus que de 25 μ environ. La basale glandulaire s'amincit fortement. Enfin, seule la partie initiale du canal excréteur est tapissée par un épithélium prismatique dont les cellules ont toujours leurs noyaux basaux, mais elles n'ont plus d'ergastoplasme; ces cellules s'aplatissent rapidement, si bien que leur hauteur atteint à peine 10 μ, dans la partie du canal intra-chélicérienne; dans la partie toute terminale du canal excréteur, les cellules de l'épithélium canaliculaire sont extrêmement plates; il est dont peu vraisemblable que le canal de la glande chélicérienne joue quelque rôle, autre que celui de vecteur du venin élaboré par le sac glandulaire.

Discussion

En somme, les caractères histologiques de la glande chélicérienne sont ceux qui ont été décrits par les classiques; seuls deux points sont à discuter, à savoir les caractères histochimiques du produit élaboré par la glande et le mode de fonctionnement des cellules glandulaires.

Les techniques que nous avons mises en oeuvre concourent pour faire admettre la dualité des produits figurés, élaborés par les cellules glandulaires. La morphologie même des produits de sécrétion est très significative à cet égard. Certaines des affinités tinctoriales observées n'ont pas obligatoirement une signification chimique précise; on sait, en effet, que l'érythrophilie ou la cyanophilie d'une structure peut dépendre de son état physique autant que de sa constitution chimique, mais l'histochimie fournit des indications formelles à cet égard: si les deux produits de sécrétion contiennent des glycoprotéines ou de mucopolysaccharides neutres (puisqu'ils sont colorables par l'acide periodique-Schiff et qu'ils perdent leur pouvoir de recolorer la fuchsine de Schiff par acétylation pour retrouver ce pouvoir par saponification), ils ne sont pas métachromatiques au bleu de toluidine, ils ne sont pas alcianophiles et sont bien colorables par la fuchsine paraldéhyde après oxydation permanganique), leur constitution protidique est cependant essentiellement différente; seul le produit érythrophile est riche en acides aminés aromatiques, alors que seul le produit cyanophile est riche en protides sulfhydrilés. En outre, seul le produit de sécrétion érythrophile contient des composés indoliques en concentration supérieure au seuil de sensibilité de la réaction au rosindole de Glenner.

Il est évidemment difficile de relier ces constatations histochimiques aux résultats des nombreuses recherches toxicologiques concernant le venin des Aranéides; en effet, les méthodes mises en oeuvre dans ce travail pourraient déceler des précurseurs, ou des substrats des principes actifs, et il n'est pas certain qu'elles montrent ces principes actifs eux-mêmes. D'autre part, les principes toxiques ne sont, à l'heure actuelle, définis que par certains caractères physico-chimiques et certains effect pharmacodynamiques. Les premiers chercheurs (31, 32) admettaient l'existence de deux principes distincts dans le venin (au moins chez les Latrodectidés), un principe hypertenseur et un principe neurotoxique; mais les recherches récentes, menées à l'aide de l'electrophorèse, permettent de révéler jusqu'à huit fractions (19). Il est cependant admis que ces fractions sont essentiellement de même nature et ne présentent que des différences quantitatives spécifiques; néanmoins, il convient de souligner que cette appréciation a été émise après une seule coloration des fractions électrophorétiques de nature protidique: celle qu'on obtient par l'amido-black acétique; il n'est pas invraisemblable d'amettre qu'une autre (ou d'autres colorations) ferait apparaîte d'autres composés.

Quoiqu'il en soit sur ce point, la pluralité des actions pharmacodynamiques du venin des ARANÉIDES est sans doute, pour une part, liée à la dualité du produit, décelable grâce à l'histochimie.

Quant au mode de fonctionnement des cellules glandulaires, la plupart des auteurs classiques (26, 33) ont cru à une fonte holocrine des cellules glandulaires, mais toute une série d'arguments s'oppose à cette manière de voir et avec Millot (12) il faut admettre que la sécrétion est du type mérocrine. En effet, on ne rencontre jamais de noyaux dans la lumière glandulaire et la recherche de débris cytoplasmiques y reste négative. Contrairement à ce qu'on voit dans toutes les glandes à fonctionnement holocrine, la glande chélicérienne des ARA-

NÉIDES ne comporte pas de zone "germinative", faite de cellules indifférenciées, riche en mitoses et destinée à prendre la relève des cellules épuisées. Le renouvellement cellulaire ne semble pas particulièrement rapide, de sorte qu'il est vraisemblable que chaque cellule parcourt un certain nombre de fois le cycle sécrétoire, allant depuis le prélèvement, dans le milieu intérieur, des matériaux nécessaires à la synthèse du venin, jusqu'à l'extrusion de ce dernier.

A côte des arguments négatifs mentionnés, il convient de signaler que les images d'extrusion des produits de sécrétion, sur les coupes de glandes fixées avec soin, ne sont pas rares; cependant les méthodes de la cytologie infrastructurale sont nécessaires pour décider du mode, mérocrine ou apocrine, de l'extrusion des produits élaborés par l'épithélium de la glande chélicérienne.

Conclusion

La coloration par l'azan de Heidenhain révèle dans le venin des araignées la coexistence de deux produits de sécrétion d'affinités tinctoriales différentes, l'un est en flaques cyanophiles, l'autre est en granules érythrophiles, de tailles variées. La sécrétion semble unique après coloration suivant Mann-Dominici, comme après coloration par l'APS ou par la fuchsine paraldéhyde; la dualité de la sécrétion apparaît après oxydation permanganique et coloration suivant Mann-Dominici. Les flaques cyanophiles sont riches en groupements sulfhydrilés. Les grains érythrophiles sont riches en acides aminés aromatiques.

Summary

In seven species of DIPNEUMONES ARANEIDS the secretion of the chelicerian glands is complex; histochemically two substances can be distinguished: one is granular, it is rich in aromatic amino-acids and in indolic compounds for it gives the alloxan-Schiff reaction, the Danielli tetrazoreaction and the rosindole reaction of Glenner; the other, which is not granular, contains sulfhydrils groups. The two substances are APS + and deprived of glycogen or acid mucopolysaccharids, they contain, probably, neutrous mucopolysaccharids for they lost their power to stain the Schiff-fuchsin after acetylation and recover that power after saponification; moreover, they are not metachromatic with toluidin blue, they are not alcianophilic and they are fairly well stained by paraldehydfuchsin after permanganic oxidation.

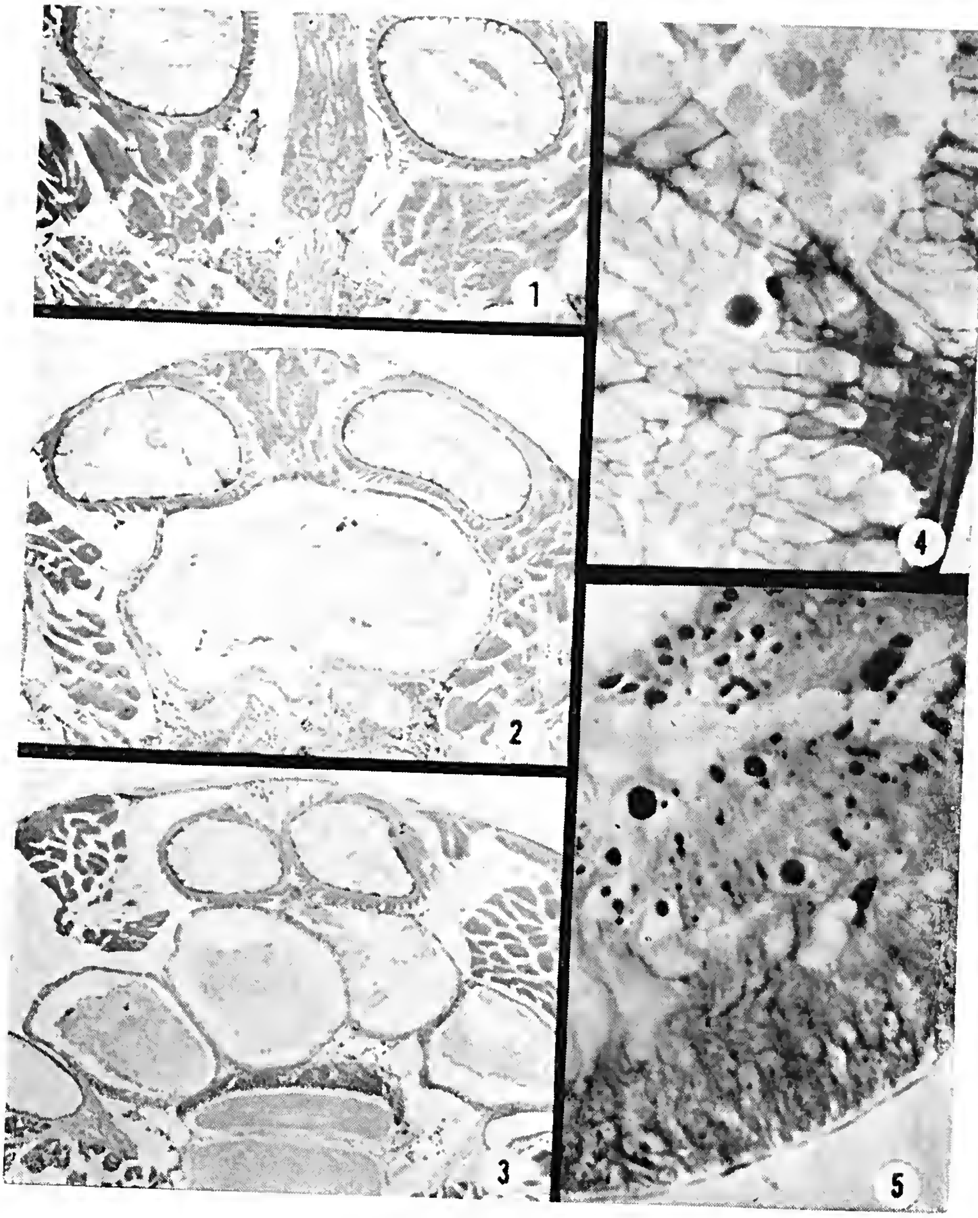

Fig. 1 à 3 — Coupe transversale du prosoma d'un mâle de *Tegenaria parietina* (Bouin-trichrome de Masson-Goldner, 90 diamètres). *Fig. 1* = coupe passant par la partie antérieure du prosoma, à ras de la base des chélicères. Remarquer les glandes chélicériennes, en position dorsale, séparées sur la ligne médiane par un raphé musculaire, la grande hauteur des cellules épithéliales et la régularité de la lumière glandulaire. *Fig. 2* = les glandes chélicériennes surplombent un diverticule thoracentérique. *Fig. 3* = la partie aborale des glandes est en rapport avec les diverticules thoracentériques et la masse nerveuse sus-oesophagienne, qui apparaît dans la partie inférieure de la photographie. *Fig. 4* — Coupe oblique du sac glandulaire de la glande chélicérienne de *Tegenaria derhami* (Bouin-coloration suivant Mann-Dominici, sans oxydation préalable, 375 diamètres, écran orange). Remarquer la basophilie des régions périnucléaires des cellules et les deux produits de sécrétion. *Fig. 5* — Coupe transversale du sac glandulaire, chez *Tegenaria derhami* (Bouin-coloration suivant Mann-Dominici après oxydation permanganique, 375 diamètres, écran orange). Remarquer l'ergastoplasme du pôle basal des cellules et la basophilie du produit de sécrétion en grains, alors que les flaques ont conservé leur acidophilie.

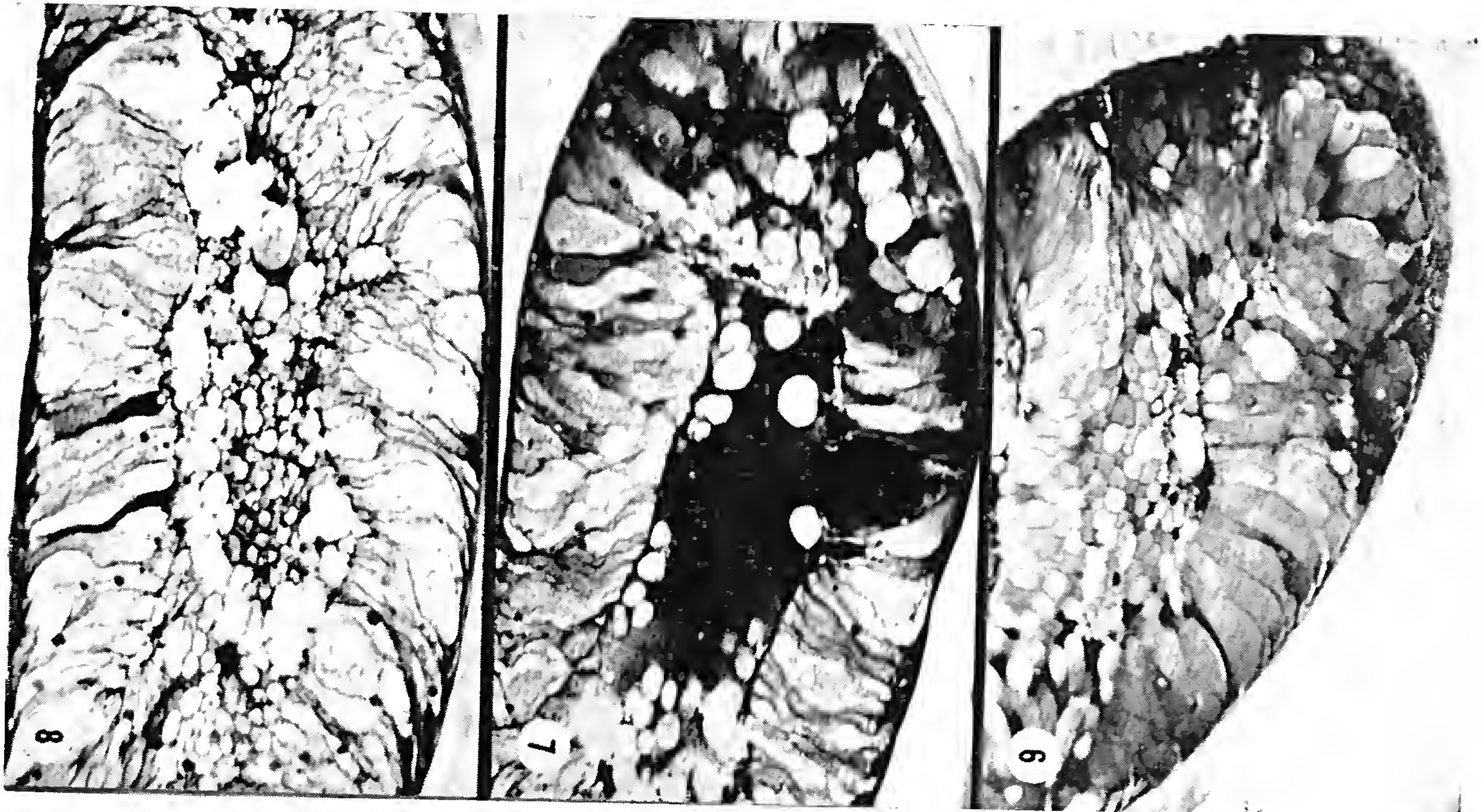

Fig. 6 à 8 — Coupes d'une même glande chélicérienne chez *Araneus diadematus* (Bouin-oxydation permanganique, coloration par la fuchsine paraldéhyde, 200 diamétres, écran vert). La *Fig. 6* montre des cellules pleines de produit de sécrétion et la *Fig. 7* une région où l'extrusion du produit a commencé: dans la *Fig. 8*, ies cellules ont éliminé la majeure partie du produit élaboré.

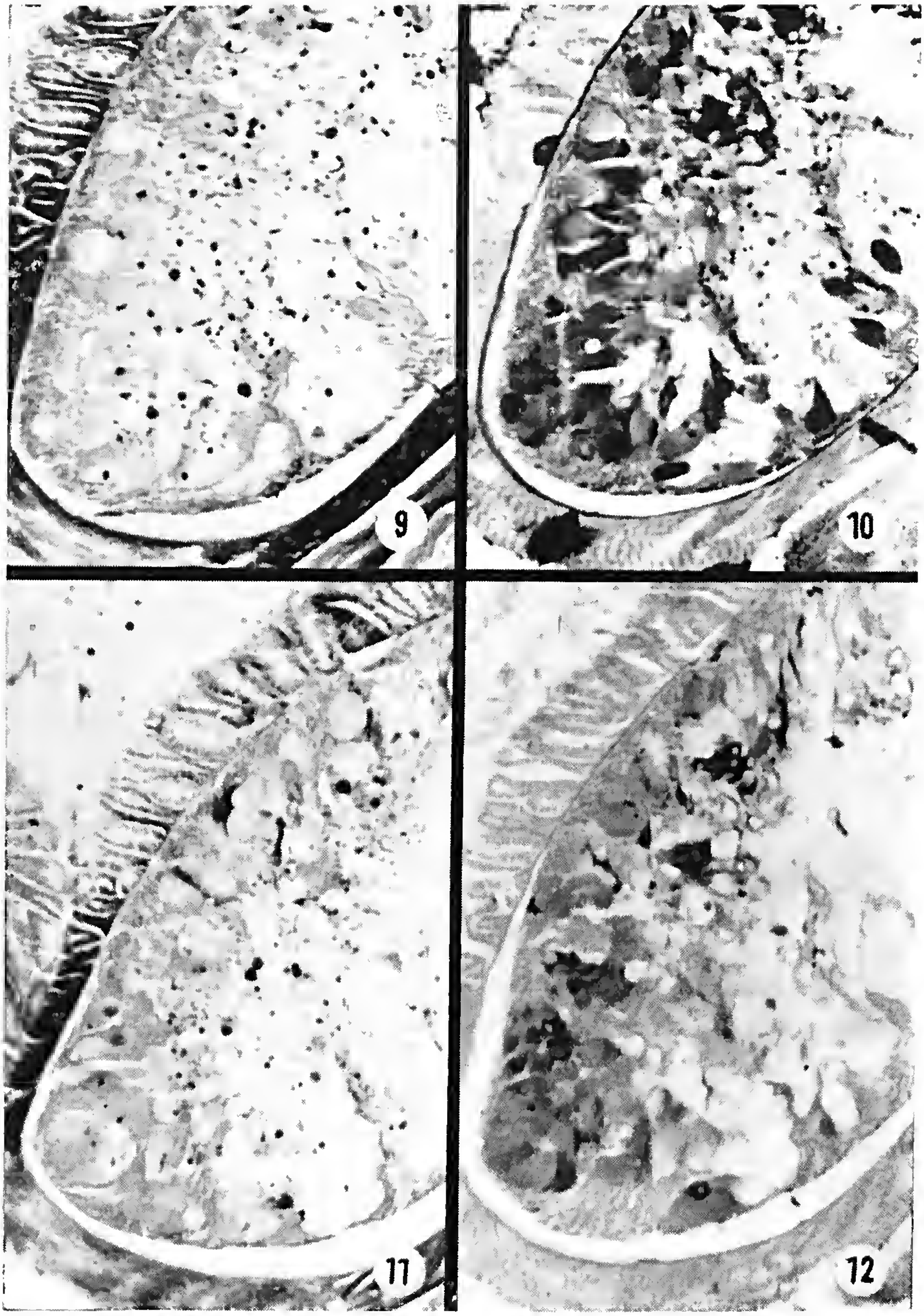

Fig. 9 à 12 — Coupes adjacentes d'une glande chélicérienne de *Tegenaria parietina* (Bouin — 200 diamètres). *Fig. 9* — coloration par l'azan de Heidenhain, écran vert: les flaques du produit cyanophile apparaissent en gris, les grains érythrophiles en noir. *Fig. 10* — APS, écran vert: les deux produits de sécrétion sont APS +. *Fig. 11* — tétrazoréaction de Danielli, écran vert: la concentration des protides est beaucoup plus forte dans les grains érythrophiles que dans les flaques cyanophiles. *Fig. 12* — ferricyanure ferrique, écran orange: seules les flaques cyanophiles sont riches en groupements sulphydrilés histochimiquement décelables, les grains érythrophiles apparaissent en négatif.

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9. *CROTALUS VEGRANDIS* KLAUBER. REDESCRIPCIÓN Y DISTRIBUCIÓN

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En 1941, Klauber (1) describió una nueva especie de serpiente de cascabel de Venezuela, basado en dos ejemplares (Holotipo: Carnegie Museum, Pittsburgh, n.º 17.384 y Paratipo: en poder del Dr. L. M. Klauber, San Diego, Calif.). Tanto el holotipo como el paratipo, fueron colectados por Harry A. Beatty en 1939. La procedencia: "Maturin Savannah, near Uracoa, Sotillo District, State of Monagas, Venezuela".

En la diagnosis de dicha especie, Klauber señala que se diferencia de *Crotalus durissus terrificus* y otras subespecies del grupo *durissus*, por tener un "pattern" peculiar, en el cual, las manchas dorsales romboidales están siempre obliteradas por la presencia de un punteado de blanco en muchas escamas dorsales. Klauber también dedujo que se trataba de una especie mucho más pequeña que *Crotalus durissus terrificus* debido a que, tanto el tipo como el paratipo, poseen los anilos de la sonaja completos (10 segmentos en el holotipo y seis segmentos en el paratipo) y, la talla de los mismos, es inferior a los *C. d. terrificus* con ese mismo número de segmentos en la sonaja.

Más tarde, en 1956, el mismo Klauber en su magnífica obra: "Rattlesnakes" (2), reconoce a *Crotalus vegrandis* como una subespecie válida del grupo *durissus*. Fué en 1958 cuando se vino a conocer un nuevo ejemplar de *C. vegrandis*, donado por un comerciante al Parque Zoológico El Pinar, Caracas, aunque sin procedencia geográfica lamentablemente. El ejemplar en referencia medía poco más de 500 mm, fué fotografiado y luego, depositado en el Museo de Biología de la Universidad Central de Venezuela.

Años más tarde, un comerciante colectó en El Tigre, Estado Anzoátegui, Venezuela, un ejemplar de 640 mm. de longitud y, lo donó a los Laboratorios Behrens, Caracas. El poblado de El Tigre dista unos 200 kms. en línea recta al oeste de la terra typica: Cerca de Uracoa, Estado Monagas, Venezuela.

Todo esto despertó un gran interés entre nosotros y nos propusimos efectuar, a fines de 1965 y principios de 1966, una investigación sobre esta serpiente de cascabel en su propia área de distribución. El presente trabajo es el resultado de dicha investigación.

Material y métodos

En este trabajo se utilizan los métodos usuales empleados en sistemática de serpientes. El material estudiado consta de una muestra de 55 ejemplares, repartidos así: 28 machos (50.5%) y 27 hembras (49,5%), procedentes de 10 localidades diferentes, incluyendo la terra típica. En la muestra hay 51 ejem-

plares adultos y apenas 4 ejemplares juveniles. También se incluyen en este trabajo los datos del holotipo y del paratipo, dados por Klauber en la descripción original. El autor tuvo el privilegio de examinar el holotipo en el Carnegie Museum, Pittsburgh, cuando visitó los Estados Unidos durante el invierno de 1961, gracias a un "leader grant" concedido por el gobierno norteamericano.

Redescripción

Crotalus vegrandis Klauber, 1941.

Crotalus vegrandis Klauber, *Trans. San Diego Soc. Nat. Hist.*, 9(30):334, 1941.

Crotalus durissus vegrandis Klauber, *Rattlesnakes, Their Habits, Life Histories and Influence on Mankind*, 1:34, 1956.

Holotipo: Carnegie Museum, Pittsburgh, n.º 17384, macho adulto, colectado cerca de Uracoa, Estado Monagas, Venezuela, por Harry A. Beatty, en 1939 (véase Fig. 1).

Fig. 1 — Aspecto dorsal del Holotipo de *Crotalus vegrandis* Klauber, 1941. (Fotografía cortesía del Dr. M. Graham Netting, Carnegie Museum, Pitts.).

Paratipo: L. M. Klauber collection, San Diego, Calif. n.º 17385, hembra juvenil, con los mismos datos de procedencia y recolección que el holotipo.

Localidad típica: Sabanas cerca de Uracoa, Distrito Sotillo, Estado Monagas, Venezuela, + 30 m. sobre el nivel del mar.

Diagnosis: Una especie que se diferencia de las del grupo *durissus*, por tener una talla muy reducida (684 mm. de largo total para el ejemplar más grande conocido hasta ahora) y un "pattern" formado por dibujos romboidales (27 a 33), obliterados por tener numerosas manchitas blancas en el ápice de muchas escamas dorsales.

Lepidosis: La escamación dorsal varía de 25-25-19 a 29-29-21 (promedio 27-27-21). Las escamas dorsales están fuertemente carenadas, especialmente en la parte anterior del cuerpo, pero las primeras y segundas hileras dorsales son lisas.

La cabeza es corta y triangularmente acorazonada. La rostral triangular, tan alta como ancha; un par de internasales triangulares y un par de prefrontales alargadas y, ovaladas posteriormente. La región frontal está ocupada por una escama grande básica o varias pequeñas y, otra corta superpuesta anteriormente; dos supraoculares normales y dos postsupraoculares. El resto de la parte superior de la cabeza está formado por escamas pequeñas y carenadas.

Las nasales divididas y los orificios nasales formados por las nasales posteriores. Hay dos loreales en cada lado; dos preoculares, dos o tres suboculares y tres postoculares. Hay tres series de escamitas entre las supralabiales y la órbita.

Las supralabiales en número de 12 a 16 (promedio 13). Hay 13 a 16 infralabiales (promedio 14). La escama mental es triangular, seguida de un par corto y un par largo de geniales.

La escamación ventral varía de 162 a 172 (promedio 165) en los machos y de 163 a 178 (promedio 169) en las hembras. La escamación subcaudal varía en los machos de 25 a 31 (promedio 27) y de 18 a 25 en las hembras (promedio 21).

Las ventrales totales varían en los machos de 188 a 201 y en las hembras de 183 a 202. Se observa un marcado dimorfismo sexual en la relación ventral-subcaudal (vease Fig. 2), pero no en las ventrales totales.

Hemipenes: Similar al de *Crotalus durissus cumanensis* *, aunque proporcionalmente más pequeño. Cada hemipene ocupa "in situ" un promedio de nueve (9) escamas subcaudales. Órganos divididos con "sulcus spermaticus" bifurcados. Parte basal del hemipene (40% del órgano) formado por espinas cortas, algo curvadas y de puntas dirigidas hacia la base; zona media y distal formada por cálices (60% del órgano) microornamentados por papilas. Final distal del ápice más o menos desnudo. El largo (comprimido) de los hemipenes varía de 20 a 24 mm.

Sonaja: En cascabel (sonaja) es pequeño y formado por una serie de segmentos que varían de 2 a 10, según la edad de los individuos.

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* En 1811, Humboldt describió dos especies de serpientes de cascabel (*Crotalus cumanensis* y *Crotalus loeflingii*, de Cumaná, Estado Sucre, Venezuela. Ambas especies fueron colocadas más tarde en la sinonimia de *Crotalus durissus terrificus*. Recientemente, Hoge, revalidó el nombre *Crotalus durissus cumanensis* para la serpiente típica cascabel de Venezuela (comunicación personal).

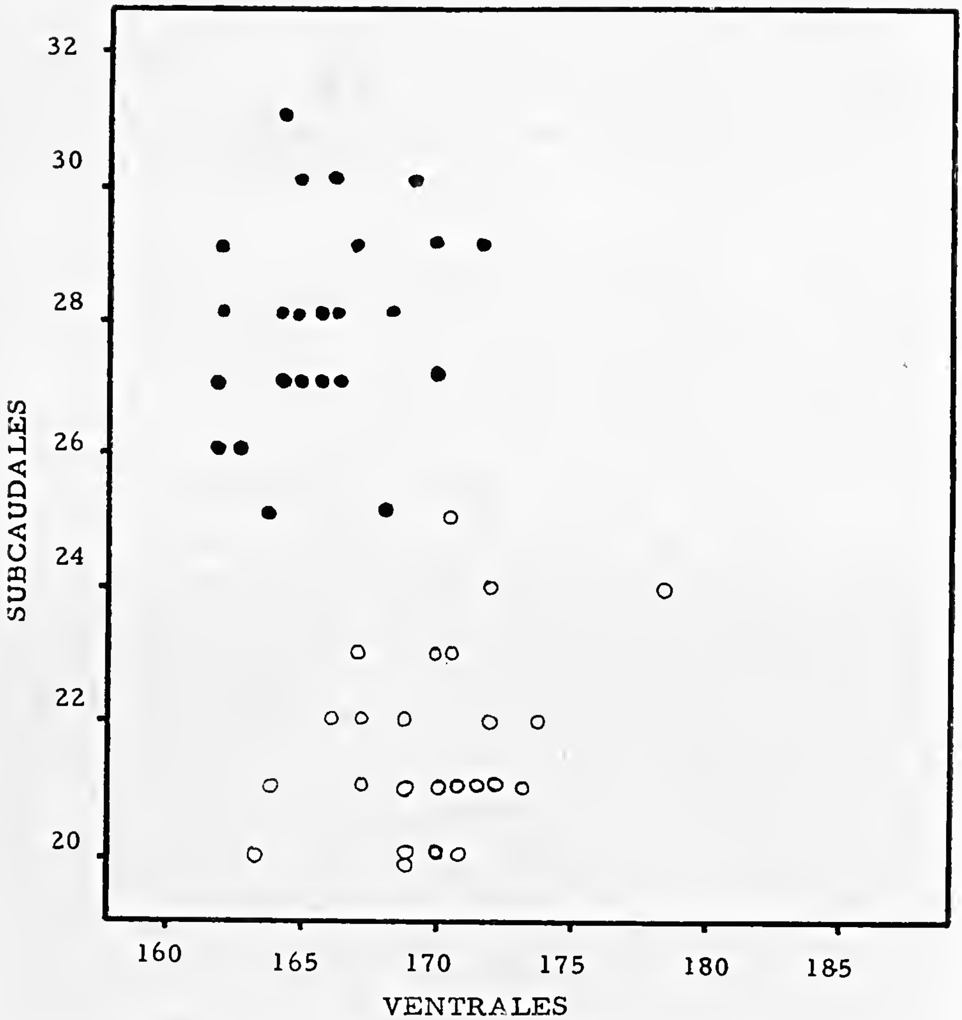


Fig. 2 — Dimorfismo sexual en el número de ventrales y subcaudales en *Crotalus vegrandis*. (Los puntos negros representan los machos y los puntos claros representan las hembras.

Coloración: La coloración de fondo varía de un gris verdoso claro a un gris pardo ceniciento, que en alcohol o formalina, se torna más pardo. En la cabeza, la rostral siempre se halla bordeada de blanco; borde de las supralabiales e infralabiales, de color blanco. Loreal y preocular superior e inferior de bordes inferiores blancos; rostro salpicado por manchitas blancas; canthales a veces manchadas de blanco. Parte superior de la cabeza con varias estrías longitudinales de manchitas blancas. A lo largo del cuerpo hay 27 a 33 rombos alargados lateralmente y, más o menos visibles. Cada rombo está formado por el blanco del ápice de escamas dorsales. Las escamas de la 1.ª y 2.ª hileras dorsales están totalmente bordeadas de blanco. Las escamas ventrales también se encuentran nítidamente bordeadas de blanco. Las geniales, gulares y primeras

ventrales son completamente blancas; cola gris obscuro en su totalidad. No se observa dimorfismo sexual en la coloración. En los ejemplares juveniles las salpicaduras blancas son más acentuadas.

Dimensiones: La talla de *Crotalus vegrandis* es bastante reducida si se toma en cuenta que en la muestra el ejemplar más grande mide: 684 mm. (macho). En el material examinado se encontraron apenas 4 ejemplares juveniles (N.os 2392, T-9, T-6) y el paratipo (N.º 17385). No se conocen ejemplares infantiles de esta especie.

Reproducción: Se encontraron 5 hembras preñadas en Diciembre 1965. Son las siguientes: N.º 2532 (3 huevos), N.º 2346 (3 huevos), N.º 2305 (3 huevos), N.º 2196 (4 huevos) y N.º 2374 (4 huevos), todos en formación.

Parece ser que el número de hijos es muy pequeño, en comparación con otras especies del género *Crotalus*.

Distribución geográfica y altitudinal: *Crotalus vegrandis* habita en los llanos y mesas de los Estados Anzoátegui y Monagas (Venezuela), en una superfície global de unos 20.000 kms. cuadrados, comprendida entre los paralelos 8° y 10° de latitud norte y entre los meridianos 62° y 65° Oeste. La región está formada por llanuras y altiplanicies (mesas) del Cuaternario. que van desde los 30 metros sobre el nivel del mar (sabanas de Uracoa(etc.) hasta unos 300 a 500 metros de altitud en las altiplanicies aluvionales (Mesas de Urica, Santa Bárbara, etc.). Las llanuras arenosas están cubiertas por gramíneas y sometidas por un clima ecuatorial de sabanas, con temperaturas medias anuales superiores a 28°C. La precipitación es más o menos abundante, pero poco efectiva. Las noches son frescas y húmedas.

Las barreras naturales en la dispersión de *Crotalus vegrandis*, las constituyen, por el norte, el Macizo Oriental; por el sur, el rio Orinoco y, por el este, el Delta del Orinoco. Los límites de distribución, por el oeste, son difíciles de precisar, aunque sabemos que esta especie no ha sido colectada más alla del meridiano 65°. Es interesante señalar que, al parecer, existe una barrera ecológica en la cuenca hidrográfica del río Unare.

Hemos localizado la periferia septentrional de la distribución de *Crotalus vegrandis*, a unos 36 kms. al sur de Maturín, Edo. Monagas, en un caserío denominado La Morrocoya (véase mapa de distribución). En este caserío ya no se encuentra *C. vegrandis* sino *C. d. cumanensis.* Un poco más al sur de La Morrocoya, las típicas sabanas de gramíneas, comienzan a sufrir transición hacia la vegetación de chaparral, que se extiende hacía el norte hasta cerca de las faldas del macizo oriental.

C. vegrandis no ha sido colectado en Uracoa ni en otros puntos de las márgenes de los ríos que cruzan el área de distribución, porque las selvas de galería de estos rios no constituyen su habitat.

Habitat: *Crotalus vegrandis* es una especie de hábitos vespertinos y se encuentra en las "matas" y sabanas que se extienden por los Estados Anzoátegui y Monagas. Al parecer, se alimenta de pequeños lagartos (*Cnemidophorus*

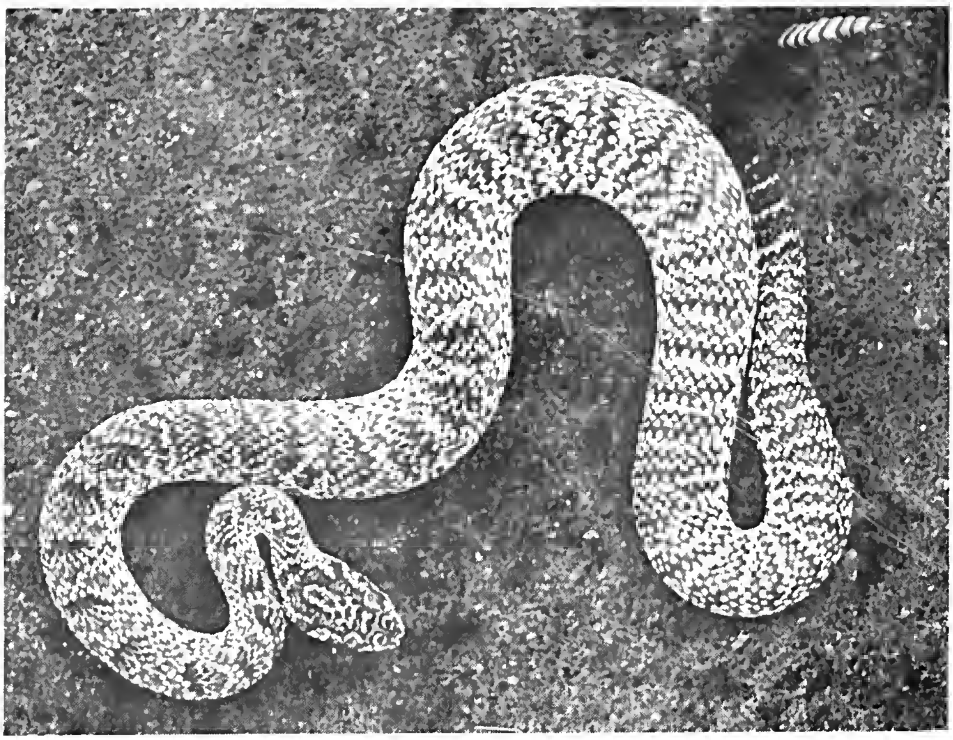


Fig. 3 — Aspecto dorsal de un ejemplar vivo de *Crotalus vegrandis* Klauber.

lemniscatus lemniscatus, Tropidurus torquatus hispidus, Hemidactylus mabouia, etc.) que abundan en la región. Los pequeños roedores (ratones, etc.) son muy escasos en estas llanuras. La coloración de *Crotalus vegrandis* es protectora (colores claros del animal sobre fondo arenoso), y le facilita la búsqueda del alimento.

Material examinado: En dos gráficos anexos (machos y hembras) se incluyen los datos de cada ejemplar estudiado y para los Museos o personas que los poseen se utilizan las siguientes abreviaturas: M.C.N.C. = Museo de Ciencias Naturales, Caracas; L.B.C. = Laboratorios Behrens, Caracas; I.M.T. = Instituto de Medicina Tropical de la U.C.V., Caracas; I.B.H. = Instituto Butantan (Herpetología), São Paulo; C.M.P. = Carnegie Museum, Pittsburgh; M.B.U.C.V. = Museo de Biología de la Universidad Central, Caracas; L.M.K. = Lawrence M. Klauber; A. R. Hoge (cedido); A. R. Lancini (cedido); A. Pons (cedido).

Mapa de distribución geográfica: En un tercer gráfico anexo, se destacan en triángulos negros, los puntos geográficos donde han sido colectados, hasta el presente, ejemplares de *vegrandis*. Las localidades de San Tomé (Edo. Anzoátegui), Santa Rosa (Edo. Anzoátegui) y Santa Bárbara (Edo. Monagas), corresponden a especímenes de la colección del Dr. Tulio Briceño Maaz, aunque no se pudo establecer la localidad exacta para cada ejemplar.

N.º	Museo
1961	M. C. N. C.
2520	M. C. N. C.
2069	M. C. N. C.
2081	M. C. N. C.
2118	M. C. N. C.
2148	M. C. N. C.
2075	M. C. N. C.
2150	M. C. N. C.
1936	M. C. N. C.
2036	M. C. N. C.
2127	M. C. N. C.
1939	M. C. N. C.
1493	M. C. N. C.
2577	M. C. N. C.
2070	A. Pons
1955	A. R. Hoge
8447	M. B. U. C. V.
2	A. R. Lancini
17384	C. M. P.
26103	I. B. H.
26101	I. B. H.
26102	I. B. H.
26104	I. B. H.
T-4	I. M. T.
T-6	I. M. T.
T-8	I. M. T.
S/N	I. M. T.
S/N	L. B. C.

MACHOS (♂) MATERIAL EXAMINADO

N.º	Museo	Localidad exacta	Colector y Fecha	L. cab (mm)	L. cola (mm)	L. corp (mm)	L. total (mm)	Dorsales	V	SC	VT	SL	IL	MD
1961	M. C. N. C.	Uverito, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	31	67	615	682	27-27-21	164	27	191	13-13	14-14	28
2520	M. C. N. C.	El Caimán, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	27	66	544	610	27-27-21	164	31	195	14-14	15-15	31
2069	M. C. N. C.	Uverito, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	31	73	611	684	27-27-21	165	28	193	13-14	16-16	28
2081	M. C. N. C.	Paso Nuevo, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	28,5	66	564	630	27-27-19	169	28	197	13-14	14-14	27
2118	M. C. N. C.	Sabanas de Uracoa, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	25,5	51	444	495	27-27-21	166	28	194	13-13	15-16	29
2148	M. C. N. C.	Sabanas de Uracoa, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	27,5	58	526	584	27-27-21	162	26	188	13-14	15-15	31
2075	M. C. N. C.	Sabanas de Uracoa, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	28	61	560	621	27-27-21	162	29	191	14-13	14-14	29
2150	M. C. N. C.	Las Pumarrosas, Distrito Sotillo, Estado Monagas.	Pedro Castillo, 3-66.	27	56	504	560	27-27-19	166	27	193	13-13	14-15	29
1936	M. C. N. C.	Paso Nuevo, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	27,5	56,5	525,5	580	27-27-19	164	28	192	12-12	14-14	29
2036	M. C. N. C.	Las Pumarrosas, Distrito Sotillo, Estado Monagas.	Angel Delgado, 3-66.	25	51	447	500	27-27-19	166	27	193	12-13	13-15	28
2127	M. C. N. C.	El Caimán, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	27,5	61	571	638	27-27-21	167	29	196	13-13	15-14	31
1939	M. C. N. C.	Paso Nuevo, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	27,5	69	569	638	27-27-21	165	27	192	13-15	15-16	29
1493	M. C. N. C.	Paso Nuevo, Distrito Sotillo, Estado Monagas.	Pedro Castillo, 3-66.	26	55	490	545	27-27-19	165	28	193	13-13	15-14	27
2577	M. C. N. C.	Sabanas de Uracoa, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	24	44,5	442,5	487	29-29-21	170	27	197	13-13	14-15	31
2070	A. Pons	Paso Nuevo, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	29,5	68	580	648	27-27-19	162	27	189	13-13	14-14	30
1955	A. R. Hoge	El Caimán, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	28,2	59	539	598	27-27-19	170	29	199	12-12	13-14	31
8447	M. B. U. C. V.	Sabanas de Uracoa, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	28	65	535	600	29-29-21	165	30	195	13-13	13-13	33
2	A. R. Lancini	Sabanas de Uracoa, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	27,5	62	549	611	27-27-21	162	28	190	14-14	15-16	31
17384	C. M. P.	Sabanas de Uracoa, Distrito Sotillo, Estado Monagas.	Harry A. Beatty, 1939	26,3	64	572	636	28-27-19	169	30	199	15-15	14-14	?
26103	I. B. H.	Las Pumarrosas, Distrito Sotillo, Estado Monagas.	Pedro Castillo, 3-66.	25	70	515	585	27-27-21	166	28	194	13-13	14-14	28
26101	I. B. H.	Paso Nuevo, Distrito Sotillo, Estado Monagas.	Pedro Castillo, 3-66.	23,5	60	470	530	27-27-19	170	29	199	14-13	15-14	29
26102	I. B. H.	Las Pumarrosas, Distrito Sotillo, Estado Monagas.	Angel Delgado, 3-66	27	70	530	600	27-27-21	167	28	195	14-14	15-15	29
26104	I. B. H.	Las Pumarrosas, Distrito Sotillo, Estado Monagas.	Pedro Castillo, 3-66.	25	65	465	530	27-27-21	166	30	196	14-13	15-14	29
T-4	I. M. T.	?	Tulio Briceño Maaz,	34	58	555	630	26-26-20	163	26	189	13-13	15-16	?
T-6	I. M. T.	?	Tulio Briceño Maaz,	26	47	363	410	28-28-20	165	28	193	13-13	16-16	30
T-8	I. M. T.	?	Tulio Briceño Maaz,	38	59	567	626	27-27-19	164	25	189	12-14	14-14	?
S/N	I. M. T.	?	Tulio Briceño Maaz,	27	53	530	583	27-27-20	168	25	193	13-13	14-14	?
S/N	L. B. C.	El Tigre, Estado Anzoátegui.	?	29	68	572	640	27-27-19	172	29	201	13-13	13-13	30

HEMBRAS (♀)

N.º	Museo	Localidade exacta	Colector y Fecha
2392	M. C. N. C.	Sabanas de Uracoa, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.
2532	A. R. Hoge	Sabanas de Uracoa, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.
2346	M. C. N. C.	El Caimán, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.
2305	M. C. N. C.	El Caimán, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.
2196	M. C. N. C.	Paso Nuevo, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.
2374	M. C. N. C.	Paso Nuevo, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.
2440	A. Pons	Paso Nuevo, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.
2459	M. C. N. C.	Sabanas de Uracoa, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.
2518	M. C. N. C.	Paso Nuevo, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.
2445	M. C. N. C.	Sabanas de Uracoa, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.
2526	M. C. N. C.	El Caimán, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.
2512	M. C. N. C.	El Caimán, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.
2517	M. C. N. C.	El Caimán, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.
2543	M. C. N. C.	El Caimán, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.
8448	M. B. U. C. V.	Sabanas de Uracoa, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.
1	A. R. Lancini	Sabanas de Uracoa, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.
T-1	I. M. T.	?	Tulio Briceño Maaz,
T-3	I. M. T.	?	Tulio Briceño Maaz,
T-7	I. M. T.	?	Tulio Briceño Maaz,
T-9	I. M. T.	?	Tulio Briceño Maaz,
T-2	I. M. T.	?	Tulio Briceño Maaz,
Ex. 17385	L. M. K.	Maturin Savannah, n. Uracoa.	Harry A. Beatty, 1939
2173	M. C. N. C.	Las Pumarrosas, Distrito Sotillo, Estado Monagas.	Pedro Castillo, 3-66.
494	M. H. N. L. S.	Temblador, Estado Monagas	?
1296	M. C. N. C.	Las Pumarrosas, Distrito Sotillo, Estado Monagas.	Angel Delgado, 3-66.
2032	M. C. N. C.	Las Pumarrosas, Distrito Sotillo, Estado Monagas.	Pedro Castillo, 3-66.
26100	I. B. H	La Dominga, Distrito Sotillo, Estado Monagas.	Pedro Castillo, 3-66.

HEMBRAS (♀) MATERIAL EXAMINADO

N.º	Museo	Localidade exacta	Colector y Fecha	L. cab (mm)	L. cola (mm)	L. corp (mm)	L. total (mm)	Dorsales	V	SC	VT	SL	IL	MD
2392	M. C. N. C.	Sabanas de Uracoa, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	23,5	30,5	416,5	447	27-27-21	172	21	193	13-13	14-14	32
2532	A. R. Hoge	Sabanas de Uracoa, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	27,5	35	490	525	27-27-21	169	20	189	13-13	14-14	29
2346	M. C. N. C.	El Caimán, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	24,5	27,5	467,5	495	27-27-21	171	20	191	13-13	14-14	30
2305	M. C. N. C.	El Caimán, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	27,5	39	519	558	27-27-19	170	23	193	14-14	14-15	29
2196	M. C. N. C.	Paso Nuevo, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	26	35	526	561	25-25-19	169	21	190	13-13	14-14	29
2374	M. C. N. C.	Paso Nuevo, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	26	39	471	510	25-25-19	171	21	192	13-13	15-14	30
2440	A. Pons	Paso Nuevo, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	26	38	521	559	27-27-19	167	23	190	13-13	16-16	30
2459	M. C. N. C.	Sabanas de Uracoa, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	26	39	451	490	27-27-21	170	21	191	14-14	16-15	31
2518	M. C. N. C.	Paso Nuevo, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	26,5	39	471	510	27-27-21	167	22	189	13-13	14-14	29
2445	M. C. N. C.	Sabanas de Uracoa, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	27	35	490	525	27-27-21	170	20	190	15-15	16-16	29
2526	M. C. N. C.	El Caimán, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	31	46	560	606	27-27-21	171	25	196	14-14	15-15	29
2512	M. C. N. C.	El Caimán, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	27	31	465	496	27-27-21	170	21	191	14-13	14-14	30
2517	M. C. N. C.	El Caimán, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	34	38,5	561,5	600	27-27-21	170	21	191	13-13	15-15	29
2543	M. C. N. C.	El Caimán, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	27	33	513	546	27-27-21	174	22	196	14-14	14-14	32
8448	M. B. U. C. V.	Sabanas de Uracoa, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	25	44	556	600	27-27-21	172	24	196	13-13	14-14	33
1	A. R. Lancini	Sabanas de Uracoa, Distrito Sotillo, Estado Monagas.	Pedro J. Ricardi, 12-65.	27	39	501	540	27-27-21	168	22	190	12-12	13-13	31
T-1	I. M. T.	?	Tulio Briceño Maaz,	26	38	492	530	27-27-19	168	20	188	13-14	15-16	29
T-3	I. M. T.	?	Tulio Briceño Maaz,	29	35	540	575	28-28-19	170	20	190	14-13	14-15	28
T-7	I. M. T.	?	Tulio Briceño Maaz,	39	40	610	650	27-27-21	166	23	189	13-14	16-15	28
T-9	I. M. T.	?	Tulio Briceño Maaz,	25	25	415	440	28-28-20	163	20	183	14-14	15-15	28
T-2	I. M. T.	?	Tulio Briceño Maaz,	27	34	498	532	27-27-20	166	22	188	15-13	16-14	29
Ex. 17385	L. M. K.	Maturin Savannah, n. Uracoa.	Harry A. Beatty, 1939	23,2	32	422	454	27-27-19	172	22	194	14-14	15-16	?
2173	M. C. N. C.	Las Pumarrosas, Distrito Sotillo, Estado Monagas.	Pedro Castillo, 3-66.	28	38	575	613	27-27-19	171	21	192	13-13	15-14	27
494	M. H. N. L. S.	Temblador, Estado Monagas	?	38	40	455	495	27-27-21	178	24	202	14-14	15-15	?
1296	M. C. N. C.	Las Pumarrosas, Distrito Sotillo, Estado Monagas.	Angel Delgado, 3-66.	26,5	33,5	448,5	482	27-27-21	169	18	187	15-13	15-15	27
2032	M. C. N. C.	Las Pumarrosas, Distrito Sotillo, Estado Monagas.	Pedro Castillo, 3-66.	27	33	503	536	27-27-19	173	21	194	13-13	14-15	27
26100	I. B. H	La Dominga, Distrito Sotillo, Estado Monagas.	Pedro Castillo, 3-66.	?	?	?	?	28-28-21	164	21	185	14-14	15-14	?

MATERIAL EXAMINADO

L. cab (mm)	L. cola (mm)	L. corp (mm)	L. total (mm)	Dorsales	V	SC	VT	SL	IL	MD
23,5	30,5	416,5	447	27-27-21	172	21	193	13-13	14-14	32
27,5	35	490	525	27-27-21	169	20	189	13-13	14-14	29
24,5	27,5	467,5	495	27-27-21	171	20	191	13-13	14-14	30
27,5	39	519	558	27-27-19	170	23	193	14-14	14-15	29
26	35	526	561	25-25-19	169	21	190	13-13	14-14	29
26	39	471	510	25-25-19	171	21	192	13-13	15-14	30
26	38	521	559	27-27-19	167	23	190	13-13	16-16	30
26	39	451	490	27-27-21	170	21	191	14-14	16-15	31
26,5	39	471	510	27-27-21	167	22	189	13-13	14-14	29
27	35	490	525	27-27-21	170	20	190	15-15	16-16	29
31	46	560	606	27-27-21	171	25	196	14-14	15-15	29
27	31	465	496	27-27-21	170	21	191	14-13	14-14	30
34	38,5	561,5	600	27-27-21	170	21	191	13-13	15-15	29
27	33	513	546	27-27-21	174	22	196	14-14	14-14	32
25	44	556	600	27-27-21	172	24	196	13-13	14-14	33
27	39	501	540	27-27-21	168	22	190	12-12	13-13	31
26	38	492	530	27-27-19	168	20	188	13-14	15-16	29
29	35	540	575	28-28-19	170	20	190	14-13	14-15	28
39	40	610	650	27-27-21	166	23	189	13-14	16-15	28
25	25	415	440	28-28-20	163	20	183	14-14	15-15	28
27	34	498	532	27-27-20	166	22	188	13-13	16-14	29
23,2	32	422	454	27-27-19	172	22	194	14-14	15-16	?
28	38	575	613	27-27-19	171	21	192	13-13	15-14	27
38	40	455	495	27-27-21	178	24	202	14-14	15-15	?
26,5	33,5	448,5	482	27-27-21	169	18	187	15-13	15-15	27
27	33	503	536	27-27-19	173	21	194	13-13	14-15	27
?	?	?	?	28-28-21	164	21	185	14-14	15-14	?

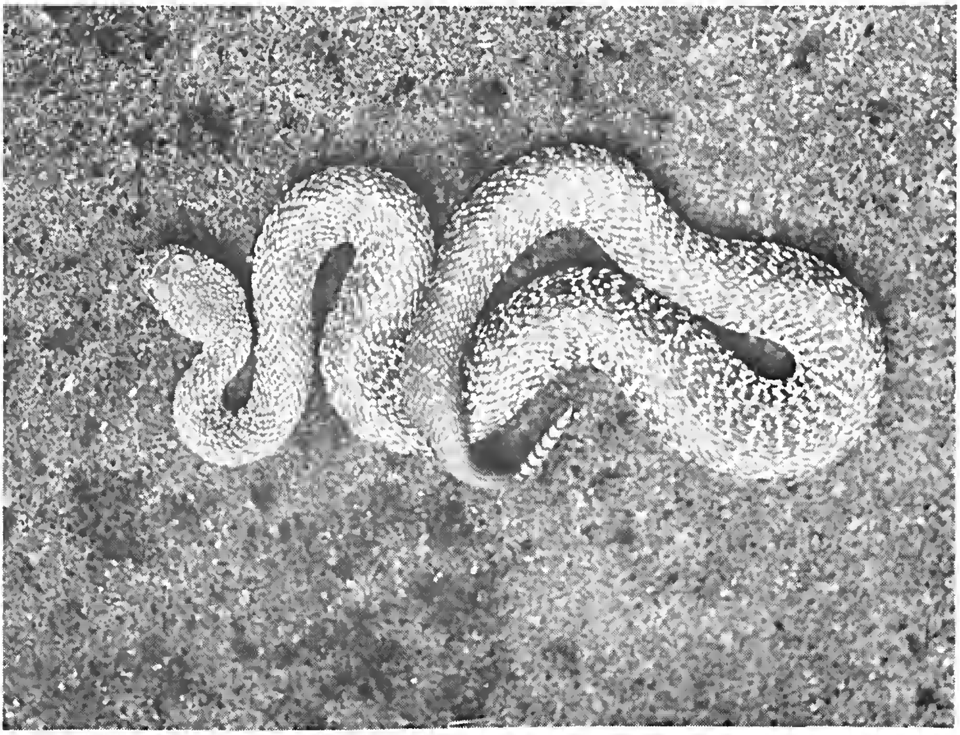


Fig. 4 — Vista dorsal de un topotipo de *Crotalus vegrandis* Klauber.

Propiedades del veneno

El veneno de *Crotalus vegrandis* es de color amarillo y cuando se deseca toma un aspecto de color cromo con marcada tendencia a frío y con tonalidades verdosas de cadmio.

Cantidad media de extracción: Se utilizó una muestra de 30 ejemplares de *C. vegrandis*, tomados al azar y, recién capturados (Una semana).

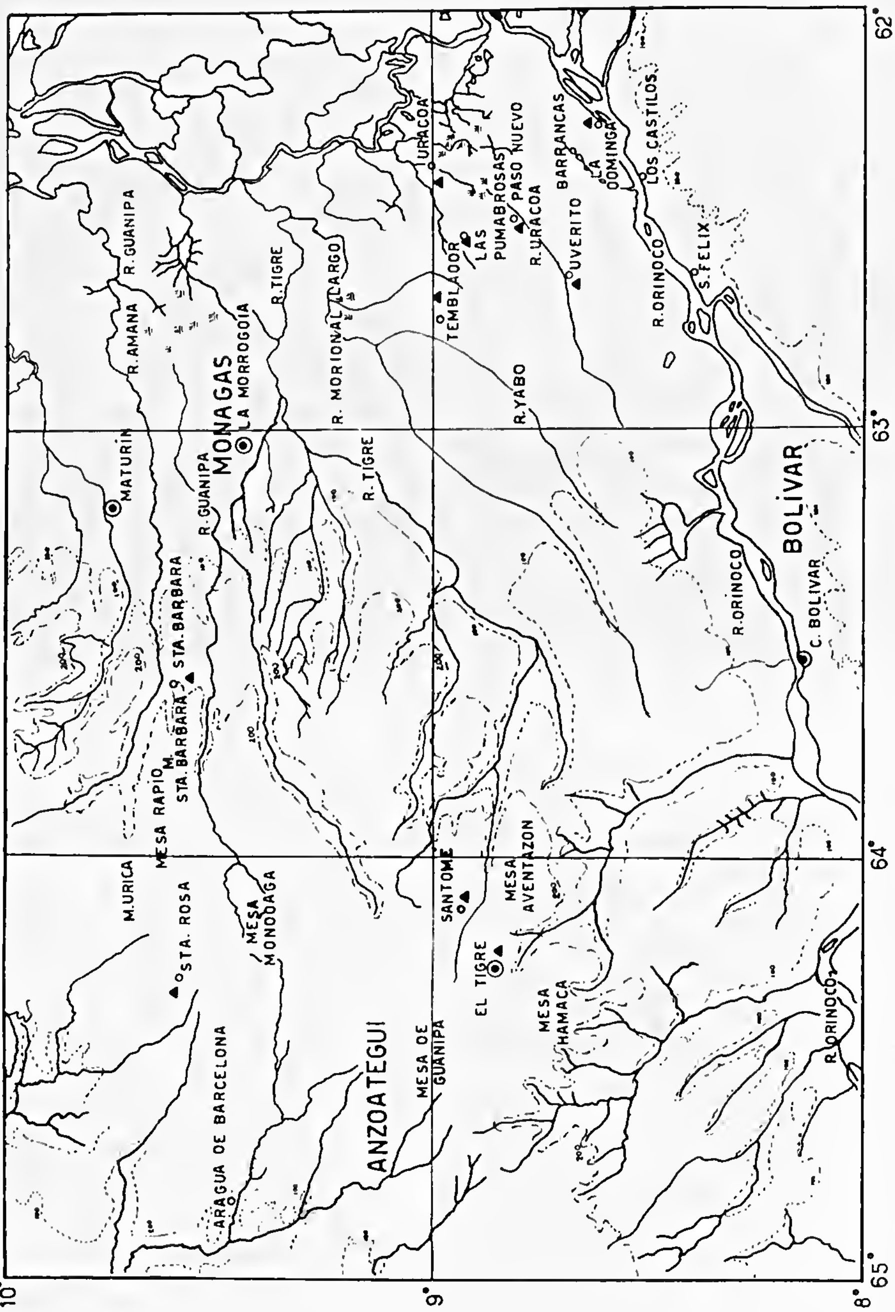
Se les extrajo el veneno por el procedimiento usual y suministraron en total 0,8 cc de veneno líquido, lo cual representa un promedio de 0.0266 cc de veneno líquido de promedio para cada ejemplar.

El veneno, desecado en presencia de $CaCl_2$, dió un producto final de 181 mgs, correspondiendo a cada ejemplar un promedio de 6.03 mgs de veneno seco por extracción.

En la muestra, los 181 mgs de veneno sólido, representan el 22.625% sobre el veneno líquido.

Determinación de la D.L.M.: Se determinó la dosis letal mínima en palomas adultas (250 a 320 gramos) y controladas durante 24 horas después de inyectadas. Se preparó una solución madre con 16,25 mgs de veneno seco y 81,25 cc de solución fisiológica isotónica, lo cual da una concentración de 1 mg de veneno por cada 5 cc de solución. Con esta solución se hizo una dilución 1/10, lo cual dió una concentración de 1 mg de veneno en 50 cc de solución, o sea, de 20 gammas por cc de solución.

REGION NOR-ORIENTAL DE VENEZUELA



▲- sitios de Coleta

DISTRIBUCIÓN GEOGRAFICA DE Crotalus vegrandis KLAUBER

Se inocularon las palomas por vía i.v. (vena del ala), con cantidades decrecientes de veneno (20, 15, 10, 5, 4, 3, 2, y 1 gamma, respectivamente) a cada ejemplar, lo cual dió como resultado la muerte de las palomas que recibieron entre 2 y 3 gammas a 20 gammas de veneno, sobreviviendo solamente los ejemplares que recibieron 1 a 2 gammas de veneno de *C. vegrandis*, en las 24 horas siguientes a la inyección. Por tanto, podemos fijar la D.L.M. del veneno de *Crotalus vegrandis* en 2 a 3 gammas.

Determinación de crotamina: Se practicó la prueba de la detección de crotamina, siguiendo los métodos empleados por Moura Gonçalves (3) y Schenberg (4). Se utilizó una solución de 0,5 mgs de veneno por cada cc de solución fisiológica isotónica.

Se inyectó un centímetro cúbico de solución, por vía subcutánea, a 8 ratoncitos blancos de laboratorio, de un peso promedio de 25 a 30 gms, no observándose parálisis del tren posterior en un lapso de 20 minutos, después de haberse practicado la inyección.Todos los ratones murieron en un plazo de 2 horas, excepto el n.º 1, que murió en 24 minutos.

Se concluyó que el veneno de *Crotalus vegrandis* es crotamino-negativo.

Discussion

Crotalus vegrandis fue descrita por Klauber, en 1941, como una especie y en 1956, el mismo autor la consideró como una subespecie válida de *Crotalus durissus*. No obstante, no hemos encontrado ejemplares intergrados en la periferia de su distribución. En el caserío La Morrocoya encontramos solamente dos ejemplares típicos *Crotalus durissus cumanensis* y ningún ejemplar de *C. vegrandis*.

Creemos que *C. vegrandis* se comporta como una especie alopátrica, que ocupa un territorio exclusivo, porque su habitat no ofrece alimento suficiente a *C. d. cumanensis* (barrera ecológica). En cambio, *C. vegrandis* se alimenta de pequeños lagartos, lo que satisface las exigencias de su talla reducida.

Al contrario, es muy probable de que *C. vegrandis* penetre en el habitat de *C. d. cumanensis*, especialmente por el oeste, y se comporte allí como una especie simpátrica. Ello se explicaría debido a que en los llanos de chaparral abundan tanto los pequeños roedores como los lagartos. Una investigación ecológica más exhaustiva contribuiría a esclarecer todo esto y permitiría establecer bien la distribución de tan interesante serpiente de cascabel de Venezuela.

Summary

There was studied a number of 50 specimens of *Crotalus vegrandis* Klauber, 1941, a rattlesnake proceeding from Uracoa, Sotillo District, State of Monagas, Venezuela, which has been known only by its holotype and its paratype. From this number 50,5% were males and 49,5% females, mostly adults. They were redescribed as a species and the following variabilities were given:

Dorsal scales:	25-25-19 to 29-29-21 (medium 27-27-21)
Upper labials:	12 to 16 (medium 13)
Lower labials:	13 to 16 (medium 14)
Ventrals:	162 to 172 (medium 165) in males
	163 to 178 (medium 169) in females

The subcaudal scales vary from 25 to 31 (medium 27) in males and from 18 to 25 (medium 21) in females.

Sexual dimorphism was observed in the relation of the ventral-subcaudal score. The relation of body-length to head-length was studied.

In this work the geographic distribution of *Crotalus vegrandis* was enlarged and the problems of distribution limits were mentioned, which are between the parallels 8° and 10° north and the meridians 62° and 65°, west longitude.

This species can be found in the savannas and alluvial plains of the States: Anzoátegui and Monagas, Venezuela. The climate of this region is macrothermic equatorial of savannas, with a medium annual temperature higher than 28°C.

Studying some properties of this snake's venom, it has been verified that it ih yellow, crotamin-negative and its M.L.D. is of 2 to 3 micrograms injected intravenously.

It is discussed if it is a species or subspecies of *Crotalus durissus* and it can be concluded that probably it is an alopatric species, since it excludes from its territory other rattlesnakes (*Crotalus durissus cumanensis*) by alimentary competition.

Agradecimientos — Debemos manifestar nuestros más sinceros agradecimientos a las siguientes personas e instituciones: Dres. M. Graham Netting, Director y Neil Richmond, Curator, del Carnegie Museum, Pittsburgh, quienes generosamente suministraron varias fotografías del holotipo y permitieron su examen; Dra. Carmen M. Antonetti, médico de la U.C.V., quien efectuó el estudio del veneno de *Crotalus vegrandis*, cuyos resultados se incluyen en este trabajo; Dr. A. R. Hoge, Jefe de la Sección de Herpetología del Instituto Butantan, São Paulo, quien, durante su permanencia en Venezuela, nos acompañó a la localidad típica y nos orientó sobremanera; Dres. J. A. de Argumosa y Guenther Berthold, de los Laboratorios Behrens, Caracas, por su amable colaboración en todo lo referente al veneno; Drs. Félix Pifano y Alejandro Mondolfi, del Instituto de Medicina Tropical de la U.C.V., por habernos permitido examinar la Colección Briceño Maaz; Dr. César Alemán, del Museo de Historia Natural La Salle, quien nos permitió examinar un ejemplar depositado en dicho Museo; Dr. Tulio Briceño Maaz, quien nos suministró la procedencia de algunos ejemplares de su colección; Oscar Alemán y Nelson Gómez, quienes colaboraron en el procesamiento de datos cuantitativos; Carlos Rivero, quien colaboró en la preparación de mapas y gráficos, Dra. Adelaida G. de Díaz Ungría, del Museo de Ciencias Naturales, Caracas, quien nos ayudó siempre con sus valiosos consejos; Pedro J. Ricardi, colector de la mayoría de los ejemplares estudiados.

Por último, debemos hacer extensivo nuestro reconocimiento al Instituto Nacional de Cultura y Bellas Artes, por sua ayuda económica en esta investigación.

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10. THE POISONOUS SNAKES OF IRAN

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During the years 1960-1965 identification of more than 10 000 snakes was made in Razi Institute. This work deals with the poisonous snakes (including OPISTHOGLYPHA) captured in various localities of Iran.

Included are 17 species and subspecies with a distribution map containing localities of the examined material; also a graph showing the number of snakes collected during a period of five years. Zoological exploration of Iran is far from complete and much taxonomic work is to be done.

OPISTHOGLYPHA

Boiga trigonata melanocephala (Annandale): Sag-mar

Head very distinct from neck, one praeocular nearly extending to the upper surface of the head; two postoculars (sometimes 2 + 1); scales smooth, more or less oblique with apical pits, in 21 rows, vertebral row enraged narrowing laterally, 8 upper labials, third, fourth and fifth entering the eye, eleven lower labials; temporals 2+2 or 2+3. Ventrals 216-229 in males, and 232 to 236 in females, subcaudals 74-88 in males and 78 to 96 in females. Yellowish olive or pale brown, with a white black-edged zigzag band along the back, or with a dorsal series of white, black-edged spots, head blackish, belly white, with or without a series of small brown spots along each side.

Total length: 1120 mm., *tail*: 170.

Localities: Kerman, Balouchestan and Sistan.

Malpolon monspessulanus insignitus (Geoffroy Saint Hilaire): Yelé-mar

Head long, angular, 8 upper labials, fourth and fifth entering the eye, 10 or 11 lower labials, two loreals, frontal very narrow, one large praeocular, reaching the frontal, temporals 2+3. Dorsal scales in 17 rows, ventrals 169-183, subcaudals 63-93 in females. Brown, olive, green, gray or bluish gray, with dark lightbrown edged spots.

Sometimes scales with blackish and whitish spots. Belly white or yellowish, punctuated with black and marked with large yellowish spots.

Total length: 1360 mm., *tail*: 290.

Localities: Azerbaijan, Central State, Kermanshahan and Khuzistan.

Malpolon moilensis (Reuss): Yelé-mar

Head long and thin, with angular conthus, 10 or 11 lower labials, 8 or 9 upper labials, the fourth and fifth entering the eye. Frontal as broad as the supraocular, one loreal as long as deep or deeper than long. One praeocular not reaching the frontal, three postoculars, temporals 2+2 or 2+3. Dorsal scales in 17 rows, ventrals 173-178 in females. Subcaudals 53-58 in females. Brown, yellow or sandy gray, spotted darker, two oblique brown or blackish bars on each side of the head behind the angle of the mouth. Belly white or yellowish.

Total length: 1020 mm., *tail*: 120.

Locality: Khuzistan.

Psammophis schokari (Forskal)

Rostral broader than deep, visible from above, neck defined, internasals much shorter than the praefrontals, the fang is followed by an interspace, one praeocular and two postoculars, nine upper labials, fifth and sixth (rarely fourth and fifth) entering the eye, 10 or 11 lower labials, temporals 2+2 or 3 (rarely 1+2). Dorsal scales in 17 rows, anal divided, ventrals 177-189 in females, subcaudals 85-125 in females. Yellowish, grayish, pale olive or reddish above, a dark streak on each side of the head, passing through the eye, a white spotted line between two brownish lines from occipital until the end of the tail, the center of the supraocular white, surrounded by brownish color, two dark lines on each side of the body. Belly usually white, yellowish, with dark spots.

Total length: 1090 mm., *tail*: 360.

Localities: Khorasan, Khuzistan and Kerman.

Psammophis lineolatus (Brandt): Tier-mar

Snout moderately prominent, grooved above and outsides. Rostral broader than deep, just visible from above, internasal much shorter than the praefrontal. The fangs not separated from other teeth by an interspace. One praeocular and two postoculars. Nine upper labials; fourth, fifth and sixth entering the eye, 10 or 11 lower labials. Temporals 2+2 or 2+3. Dorsal scales in 17 rows, ventrals 178-195, subcaudals 76-97 in females. Anal divided. Yellowish or pale gray above, with four olive black-edged stripes, the median pair extending until the top of the head, the outer pair to the nostrils, passing through the eye; a dark median streak from the interorbital region to the occipit. Belly white, dotted with gray or olive, and with one or two dark lines on each side.

Total length: 830 mm., *tail*: 200.

Locality: Khorasan.

Telescopus fallax iberus (Eichwald): Afyi-sousan

Head distinct from neck, rostral broader than deep, nasal semidivided, vertically pupil, loreal entering the eye below the praeocular, which is in contact with the frontal, 8 or 9 upper labials, third, fourth and fifth entering the eye.

Ten or eleven lower labials, (rarely 12), one praeocular, two postoculars (sometimes 2+1), scales smooth, oblique in 19 or 21 rows, ventrals 204-228 in males, 187-227 in females, subcaudals 61-67 in males, 50-64 in females; subcaudals in two rows. Anal single or divided, usually entire. Dark gray, with belly almost uniformly black, pinkish brown, belly blotches black and white. 32-40 black bars on the body, a lateral series of smaller spots alternating with dorsals, lower part speckled whitish, spotted with dark brown.

Total length: 650 mm., *tail*: 120.

Localities: Azerbaijan, Central State, Kerman-Shahan and Khorasan.

Telescopus tessellatus tessellatus (Wall): Afyi-sousan

8 or 9 upper labials; third, fourth and fifth entering the eye, 10 or 11 lower labials, the scales are in 19 or 21 rows, ventrals 215 to 261 in males, 211 to 244 in females, subcaudals 64 to 81 in males, 53 to 71 in females, anal entire or divided. It is possible, that some of the specimens here referred to as *tesselatus* are *T. martini* Schmidt, but more specimens are needed to solve the question.

HYDROPHIINAE

Hydrophis cyanocinctus (Peters): Mar-mahi

Head moderate, body elongated. Rostral slightly broader than deep; frontal much longer than broad. One praeocular and two postoculars; seven upper labials, ten lower labials, scales 41 round the middle of the body and 31 at the neck. Two specimens were examined from CHAHBOHAR (Persian Gulf). ventrals in one male: 410, and in one female: 365. Greenish olive above, with dark olive or black crossbars or annuli, broader on the back, black annuli complete and connected by a black band along the belly.

Total length: 1120 mm., *tail*: 100.

Locality: Persian Gulf.

ELAPIDAE

Naja naja oxiana (Eichwald): Kafché-mar

Eye moderate, internasal as long as or shorter than praefrontal, one praeocular, 3 or 2+1 (rarely two) postoculars, temporals 2+3 or 2+4 (2+5 or 2+6). Seven upper labials; third and fourth entering the eye, eight lower labials. Dorsal scales smooth in 21 rows. Ventrals 194 to 204 in males, 196 to 206 in females, subcaudals 57 to 69 in males, 51 to 64 in females, subcaudals all in two rows. Anal entire. Yellowish to dark brown or pale brown or gray to blackish, no marking on the hood, one or more dark crossbands on the anterior part of the belly; the young with dark rings.

Total length: 1710 mm., *tail*: 350.

Localities: Khorasan (Meshed), Gorgan.

Walterinnesia aegyptia (Lataste): Cobra

Head distinct from the neck, one praeocular resting on the third labial, one subocular, two postoculars, temporals 2+3, seven upper labials, third and fourth entering the eye; nine lower labials, scales smooth anteriorly, feebly keeled on the posterior part of the body, strongly so on the tail, 23 rows across the body, subcaudal single anteriorly, divided posteriorly. Ventrals 188 to 198 in females, subcaudals 42+1 to 47+1 in females. Characters differentiating *Walterinnesia* from *Naja* are as follows:

1 — Anal plate divided in *Walterinnesia*, entire in *Naja*.

2 — Posterior dorsal scales of body and tail distinctly keeled in *Walterinnesia*, smooth in *Naja*.

3 — Subcaudals divided and entire in *Walterinnesia*, all divided in *Naja*.

Upper surface dark brown, blackish. entire under surface dark brownish, black.

Total length: 990 mm., *tail*: 115.

Localities: Khuzistan and Fars.

VIPERIDAE

Vipera ursinii subsp: Afyi

Snout obtusely pointed, flat above, rostral as deep as broad, visible from above, in contact with two apical shields; supraocular well developed, separated from frontal by two shields. It is similar to *Vipera ursini* Renardi, but the body is more thick and the head is more distinct from the neck, nine or ten scales round the eye, five scales between the eyes, 9 upper labials. Dorsal scales in 21 rows, strongly keeled on the back. Ventrals 130 to 136 in males, 128 to 134 in females, subcaudals 22 to 33 in males, 26 to 36 in females. Anal entire. Yellowish or pale brown, reddish above, a vertebral series of more or less regular spots, transversely oval, some or all of which may be confluent and form an undulous or zigzag band, two or three longitudinal series of dark brown or black spots along the sides, the lower ones on the outer row of scales, an oblique dark stripe from the eye to the angle of the mouth. Ventrals and subcaudals with transverse series of small white spots; gray, checkered with black and white.

Total length: 420 mm., *tail*: 50.

Locality: Alborz mountains (Tehran).

A subspecies of *V. ursinii ebneri* was described from the Alborz Mountains. The validity of *ebneri* will be discussed in another paper.

Vipera lebetina obtusa (Dwigubsky): Korze-mar or Afyi

Snout rounded, rostral as deep as broad, reaching the upper surface of the snout and contact with two or three apical shields. Upper surface of head co-

vered with small subimbricate scales, which are all more or less distinctly keeled. 7 to 12 longitudinal series of scales between the eyes, supraocular narrow, usually broken up into two or more small shields, 12 to 19 scales around the eyes, two or three series of scales between the eye and the labials, upper labials 10 to 11, lower labials 12 to 14. Dorsal scales in 23 or 25 or 27 rows (usually 25 rows), strongly keeled. Ventrals 126 to 278 in males and 151 to 181 in females, subcaudals 35 to 53 in males and 39 to 51 in females. Anal entire. It seems that *V.l. schweizeri, turanica, mauritanica, deserti* and *euphratica* are nearly the same as *V.l. obtusa.* The very variable coloration and size depends upon the localities, climate and geographical conditions.

Gray, olive, or brown, with darker dorsal blotches and lateral spotting; usually grayish buff or pale brown above with dorsal series of darker spots which may stand in pairs, alternate or unite to form cross-bars and a lateral series of large dark spots or bars. A more or less distinct dark band on each side of the head, passing through the eye and than extending to the neck. Sometimes dark dots or spots on the head present; belly white, speckled (powdered) brown, with or without dark brown spots.

Total length: 1600 mm., *tail*: 200.

Localities: It is a common poisonous snake in Iran, nearly distributed in every part: Azerbaijan, Kordestan, Tehran, Lorestan, Kermanshahan, Khuzistan, Khorasan, Kerman, Balouchestan and Sistan.

Vipera xanthina subsp: Afyi alborzi

Head covered with feebly keeled scales; supraocular well developed, erectile, the free edge angular, separated from the eye by small scales. 13 or 14 (rarely 12) around the eye, two series of scales between the eye and labials, nine or ten (rarely eight) upper labials, 11 to 13 (usually 12) lower labials, 7 to 10 scales between the eyes. Dorsal scales in 23 rows strongly keeled; ventrals 162 to 171 in females, subcaudals 27 to 34 in females. Anal entire. Pale brown or grayish above, with dorsal series of somewhat lighter reddish roundish spots, which are dark-edged on the sides; these spots may be in pairs or alternative; two dark lines on the back of the head and a dark streak behind the eye, in some specimens pale brown or grayish above, with a dark brown, reddish vertebral line from the end of the head along the dorsal until the end of the tail; belly yellowish beneath, powdered with black, each shield with a transverse series of black and white spots.

Total length: 700 mm., *tail*: 35.

Locality: Alborz mountains (Tehran).

Vipera persica persica (Duméril, Bibron and Duméril): Mar-shakhadar

Snout very short and broadly rounded, supraocular "horns" above the eye present, 15 to 19 (usually 17 to 19) scales around the eye, three series of scales between the eye and labials, one series of scales between nasal and rostral,

12 to 13 (usually 12) scales between the eye. Dorsal scales 23 to 25 (usually 23) rows at mid-body, keels of lateral scales not serrated; ventrals 150 to 155, subcaudals 39 to 49. Anal entire.

Grayish or brownish or pale yellowish brown above, with four series of large dark spots, the two median sometimes confluent and forming transverse darker bars across the back; sides of the head darker brown, due to two very obscure oblique dark bars below the eye; ventral surface uniform pale straw color, sometimes whitish beneath, dotted, with dark and a lateral series of dark spots.

Total length: 850 mm., *tail*: 110.

Localities: Khorasan, Balouchestan and Sistan.

Echis carinatus pyramidum (Geoffroy Saint-Hilaire): Mar dzafari or kockmar

Snout very short, rounded, head covered with keeled scales, and very distinct from the neck, a narrow supraocular present, 10 to 12 upper labials; 11 to 13 lower labials; 15 to 19 scales around the eyes; usually two series (rarely three or one) scales between the eye and upper labials, 11 or 12 scales across from eye to eye. Dorsal scales 31 to 37 (usually 37) rows round the middle of the body, and 27 at the neck; ventrals 170 to 183, subcaudals 26 to 37 in a single row. Anal entire. Yellowish, grayish, brown or brownish gray with longitudinal series of whitish, dark-edged spots; enclosing a round dark brown lateral one, a cruciform shaped whitish mark present on the head. Belly uniform whitish, speckled with brown dots, or with small round black spots.

Total length: 660 mm., *tail*: 65.

Localities: Khorasan, Kerman, Fars, Khuzistan, Balouchistan and Sistan.

CROTALINAE

Agkistrodon halys caucasicus (Nikolsky): Afyi kafkazi

Snout obtusely pointed, slightly turned up at the end, rostral as deep as broad or broader than deep; a pair of internasals, and a pair of praefrontals; frontal as long as or little shorter than the parietal, upper praeocular separated from posterior nasal by a loreal; one praeocular, one subocular, two postoculars; loreal pit separated from labials; three large temporals, 7 or 8 (usually 8) upper labials. scales keeled in 23 rows; ventrals 149 to 166; subcaudals 31 to 56 pairs.

Yellowish, grayish, reddish or pale brown above, with darker spots forming transverse cross-bars; two lateral streaks on the back of the head; lips speckled with brown ;lower parts whitish, more or less speckled with gray or brown.

Total length: 560 mm., *tail*: 70.

Locality: Alborz Mountain (Tehran).

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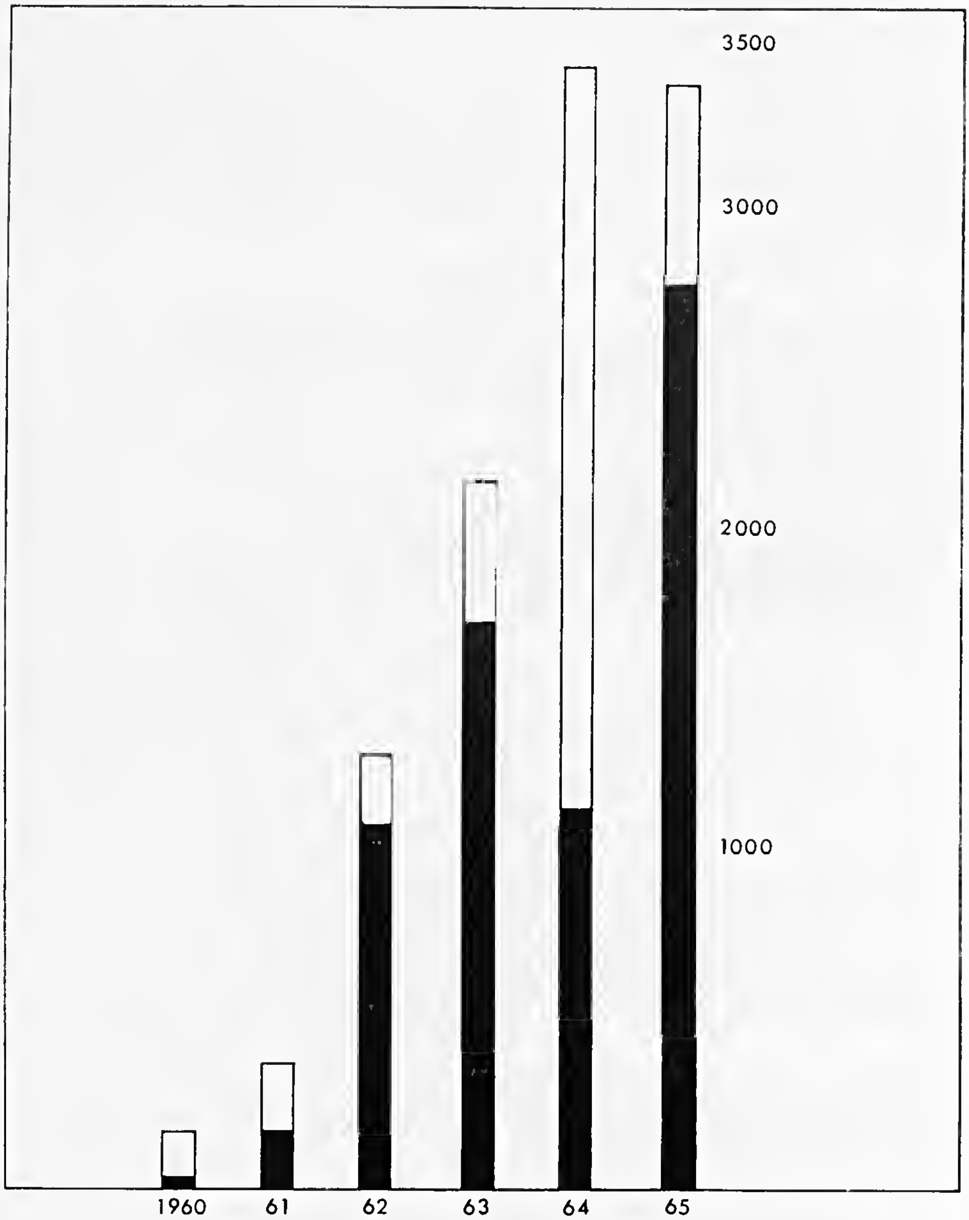


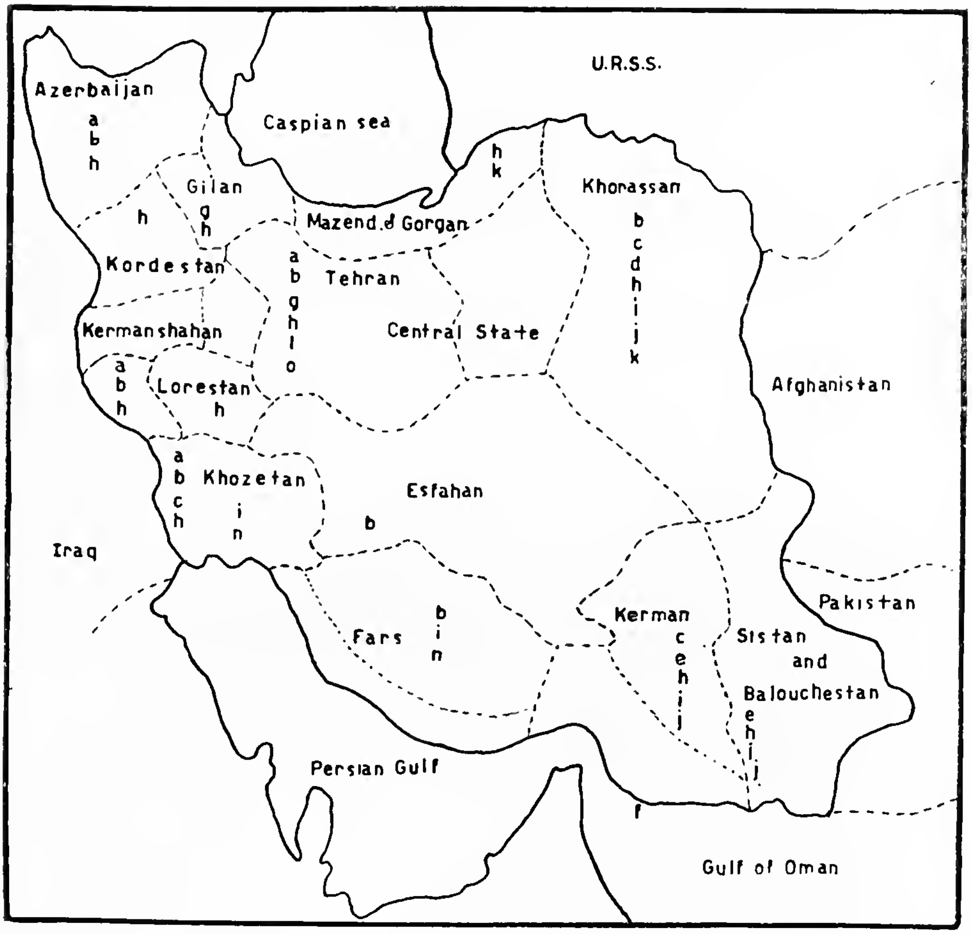
Graph showing the number of snakes collected (1960-1965)

Harmless snakes

Poisonous snakes

Each millim. 20 snakes

DISTRIBUTION OF POISONOUS SNAKES IN DIFFERENT STATES OF IRAN



a — *Malpolon*

b — *Telescopus fallax iberus*

c — *Telescopus tessellatus tessellatus*

d — *Psammophis schokari*

e — *Psammophis lineolatus*

f — *Boiga trigonata melanocephala*

g — *Hydrophis cyanocinctus*

h — *Vipera ursinii* subsp.

i — *Vipera lebetina obtusa*

j — *Echis carinatus pyramidum*

k — *Vipera persica persica*

l — *Naja naja oxiana*

m — *Vipera xanthina* subsp.

n — *Agkistrodon halys caucasicus*

o — *Walterinnesia aegyptia*

Mem. Inst. Butantan
Simp. Internac.
33(3):745-760, 1966

11. DIE SANDOTTER, *VIPERA AMMODYTES AMMODYTES* LINNAEUS

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Geographische Verbreitung

Von den Giftschlangen leben in Europa nur die zur Familie Viperiden gehörenden Viiperinae-Arten. Eine der grössten und giftigsten Arten ist die *Vipera ammodytes ammodytes* Linnaeus, die Sandotter (V.a.).

Die Heimat der Sandotter ist die Balkanhalbinsel, besonders Jugoslawien, und zwar die Provinzen Herzegowina, Dalmatien, Montenegro und das alpinische Slowenien (Abb. 1). Man findet sie auch in den westlich und nördlich angrenzenden Bergländern wie Oesterreich, Italien, Rumänien und Bulgarien. Es soll noch erwähnt werden, dass man ihr auch in den bayerischen Alpen begegnet ist. In Albanien und Griechenland wird sie von der ihr sehr ähnlichen *Vipera ammodytes meridionalis* Boulanger abgelöst. Auch in Nordafrika, in Algerien, ist die *Vipera ammodytes* gefunden worden (1, 2, 3, 4, 5).

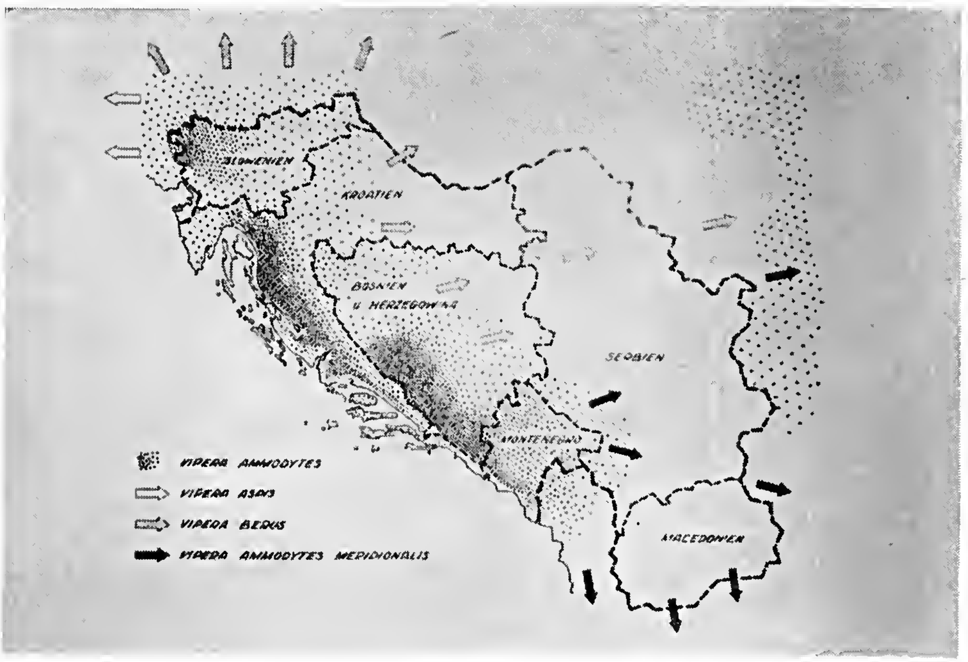

Abb. 1 — Geographische Verbreitung der *Vipera ammodytes* in Jugoslawien und angrezenden Ländern.

Die Namen *Vipera ammodytes* sowie Sandotter sind für diese Schlange unpassend, da sie auf sandigem Gelände nie vorkommt. Besser würde der Name Karstviper, *Vipera saxosa, saxea,* zutreffen, wird doch das Karstgebiet von ihr besonders bevorzugt. In der serbokroatischen Sprache gibt es für diese Viper über 200 Bezeichnungen. Die häufigste ist "Poskok" — der Springer — eine Bezeichnung, die ebenfalls nicht besonders zutrifft (6, 7, 8, 9). Diese Bezeichnung rührt von den zahlreichen Legenden über diese Schlange her, die besagen, dass sich die Schlange aus der Angriffsposition mit einem Sprung, Absprung, auf den Menschen wirft. Tomasini (6) führte in die deutsche Terminologie den Namen Modrass ein, den er der slowenischen Sprache entlichen hat (modras). Interessant ist, dass jede dieser 200 Bezeichnungen nach irgendwelchen Eigenschaften dieser Schlange gewählt worden ist. So gibt es Namen, die die Morphologie dieser Schlange hervorheben: Einmal ist es die Zeichnung, dann die Farbe oder die Aehnlichkeit mit der Umgebung. Weiter war für die Prägung des Namens die Bewegungsart, das Verhalten, die Giftigkeit und zuletzt auch das Zischen ausschlaggebend.

Morphologie

Die *Vipera ammodytes ammodytes* wird bis zu 90 cm gross, sogar Exemplare von über einen Meter Körperlänge sollen schon vorgekommen sein (10). Der Kopf dieser Schlange ist sehr charakteristisch; neben der ausgeprägten herzförmigen Kontur des Kopfes hat die Schlange and der Nase eine mit einigen Schuppen bedeckte Schnauzwarze, die oft als Horn bezeichnet wird. Daher auch der irreführende Name "Hornviper" für diese Schlange, der eigentlich der nordafrikanischen Viper *Cerastes cornutus* zukommt. Die *Vipera ammodytes meridionalis* hat ein ähnliches "Horn". Begegnet man also einer Schlange mit der beschriebenen Schnauzwarze, sei es in Europa oder Afrika, so steht man zweifellos einer Giftschlange gegenüber!

Die Schnauze wird vom Rüsselschild, Rostrale, gebildet, dann folgen links und rechts je ein Nasorostrale, die bis an den Canthus rostralis reichen. Im Gegensatz zur *Vipera ammodytes meridionalis* ist der Rostrum bei der Sandotter relativ breit, kurz und ziemlich unregelmässig in seiner Form (11).

Unter dem Nasorostrale beginnt eine Reihe von Supralabialia. Manchmal findet man zwischen dem Nasorostrale und dem ersten Oberlippenschild eine kleine Einlage, Squama praenasalis. Das Nasenschild, Nasale, ist ungeteilt. Das Nasenhorn ist, wie schon gesagt, ein aus weichem Bindegewebe bestehender Nasenfortsatz, der mit 3-4 quergelegten Schuppenreihen gepflastert ist. Das "Horn" kann fast senkrecht (aufrecht) stehen, manchmal auch etwas nach vorn oder sogar nach hinten gerichtet sein. Dieser Nasenfortsatz hat die Phantasie der Serben und Kroaten angeregt zur Schaffung einer ansehnlichen Zahl von volkstümlichen Namen für die *Vipera ammodytes ammodytes,* wobei für den Fortsatz alle möglichen Varianten und Synonima wie Nase, Schnabel, Horn und ähnliches zur Anwendung kamen.

Ueber den Augen befindet sich der Scutum supraculare, der ziemlich gross ist. Um die Augen herum befindet sich ein skleraler Ring, der aus zwei Reihen konzentrisch angeordneter kleiner Schuppen gebildet wird. Die Pupille ist, wie das für die Vipern charakteristich ist, eine vertikale Spaltpupille. Auf dem oberen Teil des Kopfes findet man manchmal einen Scutum frontale und zwei Scuta parietalia. Die untere Lippe hat in der Mitte einen Mentale und ein Kinnschild, von welchem aus sich zu beiden Seiten 4-5 Sublabialia anschliessen. Unter und

hinter dem Mentale verlaufen parallel zueinander zwei inframaxillare Schilder. Darauf folgen der Länge nach randabwärts 10-12 Reihen kleiner Schuppen, Gulares, die meistens mit 21 Reihen vertreten sind. Daran schliessen sich beim Männchen 143-160 Reihen quergelegte und beim Weibchen 141-158 Bauchschilder, Ventralia, an. Dann folgen die Subcaudalia: beim ♀ 27-36 und beim ♂ 25-44 Reihen. Die Subcaudalia findet man ab und zu von der 1.-7. Reihe zusammengewachsen an.

Die Rückenschuppen sind keilförmig, leicht abgerundet und decken den Rücken in 21-23 Längsreihen in kaudaler Richtung.

Entlang des Rückens, vom Kopf angefangen, verläuft die Zeichnung, Pictura, ein dunkelgraues bis schwarzes Längsband in Zick-Zack Form. Auch durch diese Zeichnung wurde die Phantasie unseres Volkes zur Prägung verschiedener Bezeichnungen für diese morphologische Charakteristik der Schlangen überhaupt und auch für den Namen der *Vipera ammodytes* angeregt. Die Zeichnung beginnt am Kopf mit ganz eigenartigen Figuren wie X und V oder in der Form einer Lyre. Sie verläuft den Rücken entlang (Abb. 2). Die Schuppen des Rumpfes sind klein und herzförmig. Sie decken sich wie Dachziegel in der Richtung Kopf-Schwanz.

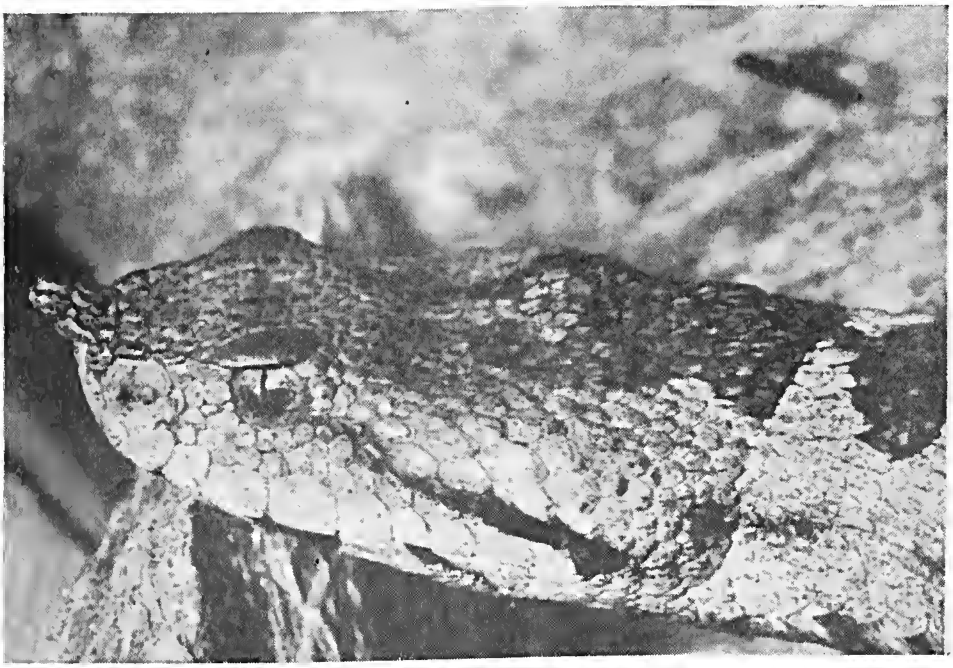

Abb. 2 — Kopf der *Vipera ammodytes ammodytes* Linnaeus.

Die Farbe der Sandotter variiert stark und wird meistens auf die Umgebung abgestimmt. In den Bergen, besonders im Karstgebiet, wo sie am häufigsten vorkommt, hat sie eine gelbbräunliche bis aschgraue Farbe; in bauxitreichen Gebieten eine braunrötliche Tönung.

Der Schwanz ist relativ kurz und endet in einer stumpfen Spitze — ein weiteres Merkmal der Giftschlangen.

Geschlechtliche unterschiede

Die Unterscheidung des Geschlechtes ist ziemlich schwierig. Im allgemeinen gilt die Regel, dass das Männchen, lat. *serpens mas*, etwas grösser ist. Die Farbe der Schuppen am Rücken ist sehr oft grau oder braun, der Bauch lichtgrau bis schwarz. Beim Weibchen ist die Zeichnung etwas verschwommen, besonders in den kaudalen Partien. Das Männchen hat sehr oft an der Unterlippe, an den Sublabialia, Pigmentflecken. Aehnliche Pigmentflecken setzen sich fort an den Körperflanken. Auch diese sind beim Weibchen weniger ausgeprägt. Die Schwanzspitze ist rötlich, korallenrot, bis gelblich.

Das eigenartige Begattungsorgan des Männchens, der paarig eingelegte, vorstülpbare Penis, in der Schwanzwurzeltasche untergebracht, kann äusserst selten beobachtet werden.

Die Paarung

Die Paarung erfolgt im späten Frühling. Auf sonnigen Felsen findet man oft Schlangenknäuel, *in semet convolvi serpentis more, serpentes circumvolutae sibi ipsae*, das Männchen und das Weibchen in der Paarung begriffen, liegen stundenlang intim aneinandergeschmiegt.

Nach vier Monaten wirft das Weibchen 6-16 vollentwickelte Junge, von 10-15 cm Grösse. Die Jungen gleichen den Alten vollkommen und sind schon vom ersten Tag ihres Lebens auf sich selbst überlassen. Sie ernähren sich durch verschiedene Käfer. Später lernen sie auch allmählich junge Mäuse und Vögel zu töten und zu verschlingen.

Der Biotop

Die Sandotter bevorzugt gebirgiges Gelände, besonders das trockene und heisse Klima in den kahlen Bergen des Karstgebirges. Dort sieht man, wie sie sich zu Knäueln vermengt, *pila serpentina*, auf dem heissen Gestein sonnen. Die Sandotter bewohnt die Berge Herzegowinas, Dalmatiens, des Montenegro. Man findet sie auch in den Bergen Serbiens, Kroatiens (das Lika-Gebiet, Istrien) und in den slowenishen Alpen. Die Abb. 3 zeigt uns das typische Gelände, wo die *V. ammodytes* haust. Meistens ist das in den Höhen bis zu 700 Metern, doch wurde sie auch schon in 1700 Metern Höhe beobachtet und gefangen genommen. In diesen Höhen ist sie wohl auch die einzige Giftschlange. Die Kreuzotter hingegen bevorzugt Täler und feuchteres Gelände wie Wiesen, Wälder, Gebüsch und die Nähe von Wasser. Damit soll aber nicht gesagt sein, dass auch die Sandotter schon in den eben geschilderten und für die Kreuzotter charakteristischen Gegenden gefunden worden ist. Sie klettert sogar auf Bäume, was für den Menschen besonders gefährlich ist. Dort hebt sie die Nester der Vögel aus und verschlingt die Jungen. Von diesem Versteck aus lauert sie auf das Vorbeifahren eines Bauernwagens, der mit Heu, Stroh oder Holz beladen ist. So wird sie dann bis in den Bauernhof verschleppt. Dort, wenn sie kein Unheil verursacht, begnügt sie sich mit Mäusejagd; ist jedoch eine ständige Gefahr für Mensch und Vieh. Mir wurde schon ein Fall mitgeteilt, wo sich eine Sandotter von einem Ast aus auf einen vorbeiziehenden Reiter warf und ihn in den Nacken biss; eine Bisstelle, die stets zu einem katastrophalen Ende führt. In Gegenden, wo diese Schlange haust, ist es also sehr gefährlich, in einem Busch nach einem

Abb. 3 — Typisches Wohngelände der Sandotter.

Vogelnest oder nach sonst etwas zu greifen. Oft tritt ein barfüssiger Hirtenjunge, sein Hund oder das Vieh auf eine im Gras oder im Geröll liegende Sandotter.

Die Sandotter pflegt sich nicht weit von ihrer Unterkunft zu entfernen. Die Behausung liegt unter einem Stein oder Felsen. Dort verbringt sie die Hälfte des Jahres, vom Herbst bis zum Frühling. Oft findet man als Zeichen ihrer Gegenwart die abgestreifte Schlangenhaut.

Im allgemeinen ist die Sandotter ein ängstliches Tier und verkriecht sich sehr schnell, sobald sie einen Menschen wahrnimmt. Erst in der Todesangst beisst sie zu. Bei gewissen Gelegenheiten zischelt die Sandotter, so z.B. bei der Verfolgung eines Opfers, in Wut oder Angst (*sibilus, stridor serpentis*).

Die Streifung

Die Sandotter streift ihre Haut, *exuvia serpentis senecta vernatio*, die eigentlich die oberste, keratinisierte Epithelschicht ist und mit der Zeit *in toto* desquamiert, mehrmals im Jahre und besonders während des Wachstums ab. Sie beginnt mit der Streifung an der Schnauze, indem sie die Haut durch absichtliche Reibung an festen Gegenständen der Umgebung zerreisst. Diesem Vorgang hilft sie durch Kriechen zwischen engen Stellen im Gestein so lange nach, bis sie schliesslich die Haut umstülpen und abziehen kann. Gelegentlich beobachtet man, dass die Sandotter sogar das Wasser aufsucht, damit die Haut weicher wird und so die Streifung schneller zustandekommt.

Bei der Betrachtung der Haut sehen wir deutlich die abgestossene Hornhaut, wodurch uns das Phänomen des starren und "hypnotischen" Blickes der Schlange verständlich wird. Gesunde Schlangen streifen die Haut im ganzen ab, während sich bei kranken die Häutung in Fetzen vollzieht.

Die Ernaehrung

Die Nahrung der Sandotter besteht aus Mäusen, Vögeln, Eidechsen, Maulwürfen und manchmal sogar aus eigenen Verwandten. In der Abb. 4 bringen wir ein Beispiel des Schlangenkannibalismus: eine Sandotter verschlingt eine

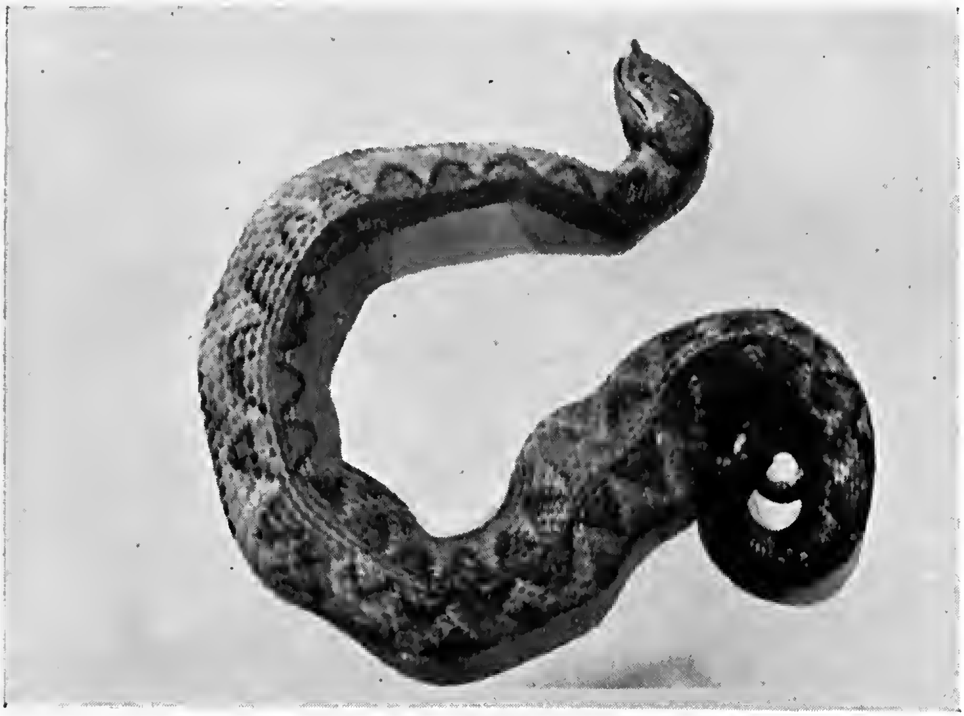

Abb. 4 — Foto eines "Kannibalen".

andere, ein durchaus nicht kleineres Exemplar. Die Röntgenaufnahme dieses Falles (Abb. 5) gibt deutlich den geschlängelten Schatten des Opfers wieder. Interessant ist indessen, dass der gierige Kannibal nach diesem Mahl bald selbst eingegangen ist. Eine ähnliche Erfahrung hat auch Dittmars gemacht (12).

Der Giftapparat

Der Giftapparat der Sandotter besteht aus der Giftdrüse, *Glandula venenosa*, mit ihrem Ausführungsgang, der in die Duplikatur der Mundschleimhaut einmündet, und dem Giftzahn, *dens venenatus viperae, telum veneficum, telum viperae*. Kathariner hat in seinen ausführlichen Studien über die Entwicklung

Abb. 5 — Röntgenbild des "Kannibalen".

und den Ersatz der Giftzähne, sowie auch über die Mechanik des Bisses, berichtet (13, 14).

Der Giftapparat dient der Schlange zuerst zur Sicherstellung ihrer Nahrung und zur Tötung der Lebewesen, die ihr als natürliche Nahrung gelten. Erst in zweiter Linie steht ihr der Giftapparat für die Abwehr und zum Angriff zur Verfügung. Die Abb. 6 zeigt uns den Schädel einer Sandotter.

Die Giftdrüse ist eine Speicheldrüse, die anatomisch der *Glandula labialis superior* entspricht (15). Auf der Mikrofotographie (Abb. 7) erkennt man deutlich die weiten *Tubuli*, deren Epithel relativ niedrig und zylindrisch ist. Der Ausführungsgang dagegen ist mit einem hohen zylindrinschen Epithel ausgestattet.

Der Giftzahn ist in eine Duplikatur der Mundschleimhaut eingehüllt, in die sich der Ausführungsgang der Giftdrüse öffnet. Von hier gelangt das Gift in die obige Oeffnung des Zahnes und fliesst durch dessen zentral gelegenen Kanal, der in eine nahe der Zahnspitze gelegene Oeffnung mündet, beim Biss tief in die Wunde hinein. Diese Gifteinspritzung gleicht fast einer künstlichen Injektion. Der Zahn wird bis zu 5 mm gross und in Ruhestellung durch ein Gelenk nach hinten gerichtet; sonst könnte die Viper ihr Maul gar nicht schliessen. Die Auspressung des Giftes erfolgt durch kräftige Kontraktion der Masseter-Muskeln.

Dem Mechanismus des Bisses liegt, wie man aus obiger, kurzer, schematisierter Beschreibung entnehmen kann, ein sehr kompliziertes durch eine Anzahl von Reflexen bedingtes Muskelspiel zugrunde.

Abb. 6 — Schädel einer Sandotter.

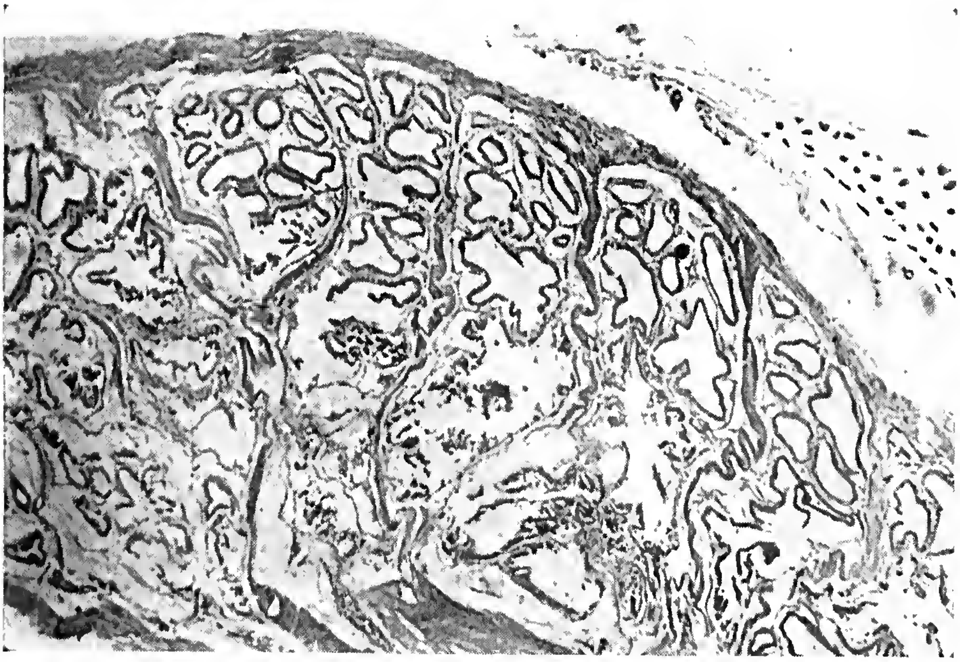

Abb. 7 — Mikrofotogram der Giftdrüse der *V. ammodytes*.

Die Sandotter beisst in Ruhestellung meist nur zur Abwehr. Falls sie ihr Opfer verfolgt, kriecht sie ihm langsam nach, um aus einer güngstigen Stellung heraus zuzupacken. Eine satte Sandotter kümmert sich überhaupt nicht um eine weisse Maus im Käfig, die sich sogar vor der Nase herumtummeln kann.

Beim Angriff wirft die Viper den Kopf mit grosser Wucht vorwärts, öffnet das Maul, richtet die Giftzähne auf, beisst zu, zuckt mit dem Kopf rückwärts und lässt dann das Opfer wieder los. Sie bezweckt damit die Erweiterung der Stichwunde und die passive Auspressung des Giftes aus der Duplikatur in den Zahnkanal. All das geschieht blitzartig, mit einer Fertigkeit, welche man der kriechenden Natur der Schlange nicht zutrauen würde. Das gebissene Opfer, die Maus z.B., läuft nach dem Biss winselnd davon und sucht ihr Heil in einem Versteck. Doch das Gift wirkt rasch und tödlich. Die Sandotter weiss das und kriecht dem Opfer langsam nach. Dann beginnt die dramatische Mahlzeit. Das Opfer wird vom Kopf her angefasst und langsam und schubweise verschlungen. Es ist fast unvorstellbar, wie weit die Schlange ihr Maul aufreissen kann, und was für ein grosses Opfer sie zu verschlingen vermag. In spätestens 20-30 Minuten sieht man nur noch einen stark erweiterten Teil des Schlangenleibes in Form einer länglichen Kontur und als letzte Spur den Schwanz der Maus, der noch aus dem Maul ragt. Nach solch einem Schmaus wird die Schlange träge, für die Umwelt ziemlich uninteressiert und kaum noch angriffslustig. Ich habe auch schon beobachtet, dass eine sehr hungrige Sandotter sogar zwei Mäuse hintereinander verschlungen hat.

Der Schlangenfang

Für den Schlangenfang gibt es eine Anzahl verschiedener Geräte (siehe Abb. 8), welche sich der Mensch in seinem milenischen Ringen mit den Giftschlangen konstruiert hat (16). Meistens sind es verschiedene Modifikationen von Spaltzangen und Holzgabeln. Die Schlangen wurden jedoch am häufigsten mit einer Keule oder einem Stock getötet. Mit dem Schuhabsatz eine Schlange zu töten, gehörte schon zu den Heldentaten.

Die Sandotter wird von der stets fortschreitenden Zivilisation mehr und mehr verdrängt; sie flüchtet in die Berge, in die Höhe. Heute ereignen sich Schlangenbisse beim Menschen sehr selten, etwas häufiger beim Vieh.

Die Sandotter war in den besagten Gegenden jahrzentelang eine wahre Plage für die Bevölkerung. Während der österreichischen Okkupationsherrschaft in den Gebieten Bosniens und Herzegowina wurden von deren Administration sogar eine Belohnung für jeden überbrachten Schlangenkopf ausbezahlt. Laut damaligen Statistiken wurden in den Jahren 1907-1911 780 Menschen gebissen, wovon 40 starben und 53.378 Stück Vieh gebissen, wovon 10.712 Stück eingingen. In der erwähnten Zeitspanne wurden den Behörden 790.642 Schlangenköpfe übergeben; eine imposante Zahl (1). Um so viele Schlangen zu fangen, bedarf es Geschicklichkeit und guter Fanggeräte, vor allem aber ein tapferes Herz. Tapferkeit schon deshalb, weil sich die Menschen im allgemeinen vor Schlangen fürchten, sogar vor toten Schlangen. Wie überall in der Welt ist die Schlange auch in diesen Gebieten von viel Mystik umgeben.

Seitdem die Schlangen zur Giftentnahme massenhaft gefangen werden, sind auch die Fanggeräte wieder in die Mode gekommen und in ihrer Ausführung verfeinert worden. Die Schlange muss unversehrt gefangen werden, da nur unbeschädigte Exemplare angekauft werden.

Obwohl die Schlangenjagd und der Schlangenfang ein gefährlicher Beruf sind, liefert er den Bauern und Hirten jener Gegenden ein beträchtliches Nebeneinkommen. So wurde im Laufe der Zeit eine Plage in einen gewissen Segen umgewandelt.

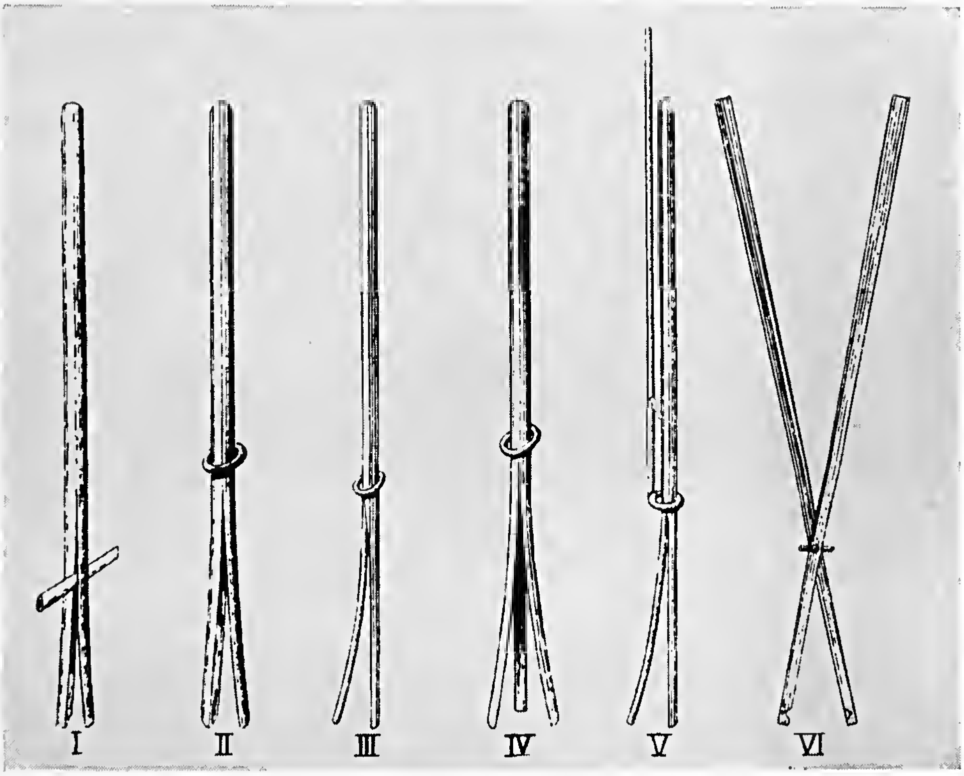

Abb. 8 — Die primitiven Fanggeräte.

Die Sandotter in der Gefangenschaft

In den Terrarien werden die Sandottern in Holz- oder Metallkäfigen, mit Glas kombiniert, gehalten. Die Temperatur muss stets auf 22-26°C bleiben, wenn die Schlangen immer frisch, gesund und rege sein sollen. Das ist die Voraussetzung für eine ökonomische Giftausbeute. Oft werden am Plafond der Käfige starke Glühlampen angebracht, die den Schlangen Sonnenschein vortäuschen sollen. Tatsächlich schlängeln sich die Vipern unter den Lampen und bilden dort Schlangenknäuel und -teller. Damit sie sich noch wohler fühlen, legt man trockene Baumäste oder frische Fichtenäste sowie auch eine Schüssel mit Wasser in die Käfige.

Die Sandotter nimmt in der Gefangenschaft zuerst nur selten Nahrung. Einige verharren in stetem Hungerstreik. Solche Exemplare leben monatelang, ja bis zu einem Jahr nur von Wasser. Selbstverständlich magern sie sehr ab; eine solche Sandotter erkennt man an der stark gerunzelten Haut am Rücken. Die andern, welche sich mit der neuen Lebensweise abfinden, bekommen durch-

schnittlich alle 14 Tage eine Maus. Das Wasser wird täglich gewechselt. In der Gefangenschaft lebt die Sandotter im Durchschnitt 2-3 Jahre.

Ich habe wiederholt versucht, die Jungen, die in unserem Institut in Gefangenschaft geboren wurden, aufzuziehen, jedoch ohne Erfolg. Die Jungen nehmen in der Gefangenschaft keine Nahrung zu sich, auch wenn man ihnen ihre natürliche Nahrung anbietet. Der Versuch, sie künstlich mit Käfern, Mehlwürmern u.a. zu füttern, scheiterte ebenfalls: entweder würgten sie die mit etwas Gewalt verabreichte Nahrung zurück, oder sie starben am nächsten Tag ohne anatomischen Befund! Auch das Einführen der Milch oder einer nahrhaften Brühe mittels einer Sonde konnte diese Wesen nicht länger als drei Monate am Leben erhalten.

Die Giftentnahme

Die Giftentnahme geschieht in zweiwöchentlichen Abständen; die Abb. 9 zeigt uns diesen Vorgang. Die Schlange wird mit dem Daumen und Zeigefinger der linken Hand dicht hinter dem Kopf angepackt. Im Zorn öffnet sie das Maul, vor welches man eine Petrischale hält. Mit dem Rand der Schale werden nun die Zähne berührt. Durch diesen Reiz wird reflektorisch das Giftausstossen bedingt, ein bis zwei Tropfen, in Form einer gelblich zähen Flüssigkeit, lässt man aus jedem Zahn in die vorgehaltene Petrischale fliessen. Die das Gift enthaltenden Schalen werden nachher in einem Exsiccator mit Chlorcalcium unter Vakuum getrocknet. Im trockenen Zustand hat das Gift eine glitzernde zitronengelbe Schuppen- und Pseudokristall-Beschaffenheit. Ein weisses Gift, wie es Kornalik und Master kürzlich beschrieben haben, konnten wir nie beobachten (17).

Abb. 9 — Giftentnahme.

Die Sandotter gibt bei einer Giftentnahme ca. 20-40 mg Gift ab (Trokensubstanz). Diese Schwankungen sind dem Gesundheitszustand, der Grösse der Schlange und der Saison unterworfen. An heissen Sommertagen sind die Erträge am ausgiebigsten.

Die Haeufigsten Erkrankungen der Sandotter

In der Gefangenschaft erkranken die Schlangen wahrscheinlich am häufigsten durch die nicht immer sanften Manipulationen der Schlangenwärter bei der Giftentnahme, wobei ab und zu ein zahn abgebrochen wird. Dadurch kommt es leicht zu Infektionen der Zahnwurzel und auch der ganzen Mundschleimhaut. Oft finden wir in dem entnommenen Gift abgestossene oder abgebrochene Giftzähne. Eine so erkrankte Schlange zeigt uns die Abb. 10, die nicht nur

Abb. 10 — Kopf einer erkrankten *V. ammodytes*: zahnlos und geschwollen.

eine durch die Entzündung geschwollene und livide Mundschleimhaut, sondern auch einen durch die Schwellung deformierten Kopf aufweist. Das Mikrofotogramm der Abb. 11 zeigt einen Schlangenblutausstrich, in dem man deutlich Streptokokken erkennt. Leider haben wir die Streptokokken nicht näher identifizieren können. Das Blutbild stammt von der Schlange, welche die oben geschilderte Krankheit hatte und wahrscheinlich an den Folgen einer Sepsis einging. Burtscher (18) hat einen ähnlichen Fall beschrieben, in dem er einen zur Gruppe des Bacillus liquefaciens gehörenden Keim für den möglichen Erreger hält (Abb. 11).

Die Schlangen tragen an ihrem Körper sehr oft Milben. Bei den Sektionen findet man im Darm manchmal Bandwürmer.

Abb. 11 — Mikrofoto des Blutes einer an Streptokokkensepsis eingegangenen Sandotter.

Der biss der Sandotter

Der Schlangenbiss bei Mensch und Tier ereignet sich fast immer unerwartet und blitzschnell. Gefährdet sind die Bauern, Hirten, Touristen und spielende Kinder, die Vogelnester ausheben oder ähnliche Streiche ausüben. Das Vieh ist beim Weiden gefährdet, ebenso der Hund (besonders der Schäfer- oder Jagdhund). Die bevorzugten Bisstellen beim Menschen sind die Beine, besonders der Fuss, ferner auch die Hände. Nach dem Biss sieht man zunächst nur zwei winzige, 5-6 mm voneinander entfernte Einstiche, die etwas bluten. Der Allgemeinzustand des Befallenen verschlimmert sich aber zusehends. Das gebissene Glied schmerzt, schwillt an und wird rot. Häufig sieht man die roten Ausläufer der entzündeten Lymphbahnen. Der Patient weist eine Blässe der ganzen Haut auf, zittert, bekommt Angstzustände und Schweissausbrüche, hat beschleunigten weichen Puls und bald darauf folgt der Kollaps. Der Atem wird kurz, schnell und mühsam. Vielfach weint der Patient; auch andere Symptome einer gewissen Reizung der Psyche sind feststellbar. Es ist wahrscheinlich nicht gerechtfertigt, diese Angstzustände als simple Folge des überstandenen Schreckens aufzufassen. Nach einigen Stunden wird das Oedem enorm, der Umfang des betroffenen Gliedes kann sich verdoppeln. Rings um die Bisstelle sieht man rötlichblaue, dunkle Flecken, verursacht durch den Blutaustritt ins Gewebe. Das Glied ist kalt, und wenn dem Patienten nicht fachgemäss geholfen wird, nekrotisiert die Umgebung der Bisstelle oder sogar das ganze Glied, vor allem aber die distalen Teile einer Extremität. Deswegen sind oft Amputationen notwendig. Das Allgemeinbefinden bessert sich nur langsam. Erfolgt der Biss ins Gesicht, Hals

oder Nacken, setzt der Tod infolge eines sich akut entwickelnden Hirnödems sehr rasch ein. Gelangt das Gift unglücklicherweise direkt in die Blutbahn, so verschlimmern sich die oben beschriebenen allgemeinen Symptome und der Tod tritt unter Atemstillstand als Folge der Lähmung des Atemzentrums ein. Doch sind Todesfälle glücklicherweise sehr selten. Oftmals gelangt nicht das ganze Giftquantum in die Wunde, weil die Giftzähne die Kleidung des Betroffenen durchdringen müssen und somit einen Teil des Giftes abfangen. Abb. 12 zeigt uns eine Frau, eine Bäuerin aus dem dalmatischen Karstgebiet, welche infolge eines Schlangenbisses und verspäteter Behandlung ihren rechten Arm bis auf einen restlichen Oberarmstumpf amputieren lassen musste. Es war schon eine weitgehende Nekrose des Armes eingetreten, verbunden mit einer Infektion und drohender Sepsis. Eine Infektion der Wunde ist nämlich häufig zu beobachten. Das rasche Fortschreiten der Infektion ist durchaus verständlich, wenn man bedenkt, dass das Gift der Sandotter reich an Hyaluronidase ist. Dem letzteren ist zu verdanken, dass sich das Oedem so schnell entwickelt und so enorme Dimensionen annimmt.

Abb. 12 — Bäuerin, der infolge des Bisses einer *V. ammodytes* der rechte Arm verloren ging.

Die Therapie des Schlangenbisses

Die Therapie richtet sich heute nach der einzigen und bewährten Methode. die möglichst rasche Einspritzung von antitoxischem Serum, am besten homologem Antitoxin. Tatsächlich ist es in den bedrohten Gebieten heute möglich, dem Verunglückten sofort Serum zu verabreichen. Es hat sich als praktisch und nützlich erwiesen, dass nicht nur jede Apotheke, jeder Arzt und Veterinär, sondern auch der Lehrer, Pfarrer und Agronom in diesen Gebieten wenigstens eine Ampulle Serum hat oder Bescheid weiss, wo es sicher und rasch zu beziehen ist. Auch die Touristen haben sich die Gewohnheit angeeignet, in ihrem Rucksack eine Ampulle antiviperines Serum und eine sterile Injektionsspritze mitzutragen.

Die anderen therapeutischen Mittel verdienen meistens nur historisches Interesse. Was man bei einem Verunglückten nebst oder auch ohne Serum tun kann und soll, ist die Bekämpfung des Kollapses, allerdings nicht mit reichlichen Gaben von Alkohol, wie es früher der Fall war. In grösseren Mengen verabreicht, fördert er bloss den Kollaps. Sofortige Erweiterung und Oeffnung der Bisstelle oder das Aussaugen des Giftes mit dem Mund hat nur einen relativen Wert. Das beste und sicherste Mittel bleibt, den Erkrankten mit oder ohne sofort verabreichtem Serum schleunigst in ein Spital einzuliefern.

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12. ESTUDO CITOLÓGICO E PONDERAL DO TESTÍCULO DE *CROTALUS DURISSUS TERRIFICUS* DURANTE O CICLO REPRODUTIVO ANUAL

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Os trabalhos sôbre o ciclo de maturação sexual das serpentes das regiões temperadas são, relativamente, escassos. Em tal assunto foram consultadas as publicações fundamentais de Volsöe (1), Fox (2) e H. Saint Girons e colaboradores (3, 4). Naquilo que diz respeito ao interessante campo de pesquisa dos ofídios brasileiros, não se conhece absolutamente nada sôbre o assunto. Foi com o fim de preencher a falta de dados, nesse campo de estudos, que colhemos os testículos de uma série de indivíduos da cascavel, *Crotalus durissus terrificus* (Laurentius) durante os anos de 1964 e 1965, no Biotério Experimental de Serpentes Peçonhentas do Instituto Butantan. Em cada mês do ano, foram colhidos cêrca de 10 indivíduos. Os testículos eram pesados e fixados em Bouin. Cada serpente foi pesada, individualmente e o comprimento total do seu corpo era medido. Ante os dados obtidos foi analisada a variação anual do testículo da seguinte maneira:

a) Estudo histológico da atividade espermatogenética;

b) Em cada grupo de indivíduos colhidos, no mesmo mês, foi determinada estatìsticamente, a regresão entre o pêso dos testículos e o comprimento total do corpo, considerando-se êste último parâmetro, como aquêle correspondente, aproximadamente, à idade do indivíduo;

c) Foi determinado o coeficiente de correlação entre o pêso testicular e o comprimento corporal dos indivíduos de cada grupo mensal.

Os resultados obtidos dêsse modo, estão apresentados na Tabela I e no gráfico da Figura 1. Infelizmente, no momento da colheita do material não foi conseguido o lugar de procedência da serpente, informação essa que poderia elucidar alguns dados anômalos desta pesquisa. Apesar disso, pela análise dos dados obtidos, chegamos às seguintes conclusões:

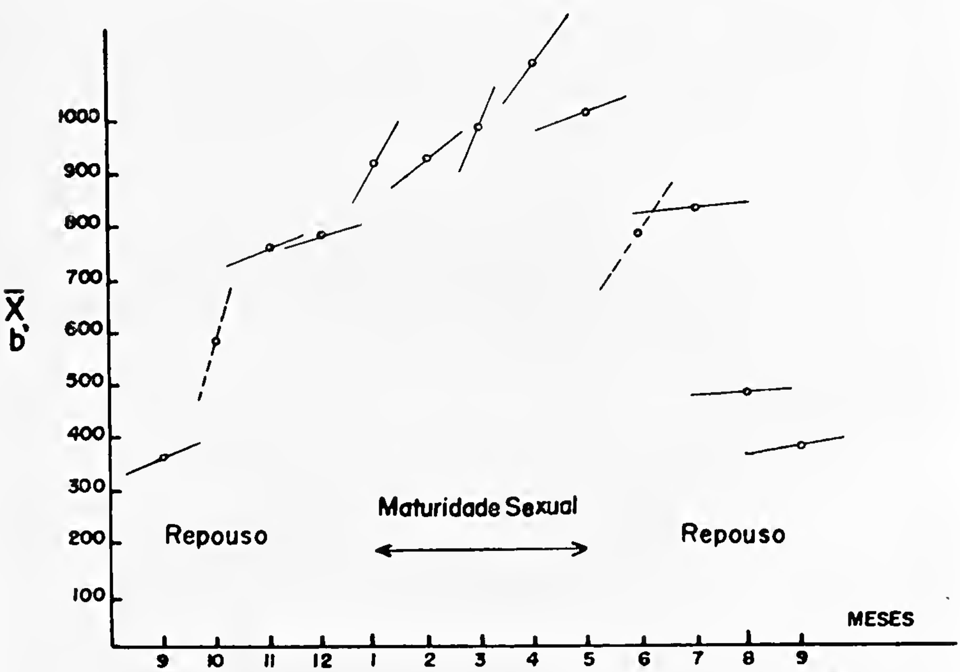
1. O pêso do testículo (média de mais ou menos 10 indivíduos em idades diferentes) varia durante o ciclo anual de um mínimo de 377 mg em Setembro de 1964, alcançando o máximo de 1106 mg em Abril de 1965 e reduzindo-se, novamente ao valor de 397 mg em Setembro de 1965;

Trabalho realizado com o auxílio do Fundo de Pesquisas do Instituto Butantan.

TABELA I

Valores da média do pêso do testículo ($\overline{Y}$), do comprimento do corpo ($\overline{X}$), do coeficiente de regressão (b'), do coeficiente de correlação (r) e do número de indivíduos (n.). O coeficiente de regressão (b') foi calculado na base de que ambas variáveis estão sujeitas a êrro.

		$\overline{X}$	$\overline{Y}$	b'	r	n
Setembro	1964	86,10	377,5	11,19	0,111	10
Outubro	"	82,83	595	84,44	0,577	6
Novembro	"	84,1	769	10,51	0,177	10
Dezembro	"	87	789,67	6,80	0,473	15
Janeiro	1965	86,2	923	46,67	0,457	10
Fevereiro	"	85,81	929	27,09	0,367	11
Março	"	87,8	987	58,47	0,664	10
Abril	"	86,8	1106,5	33,94	0,497	10
Maio	"	87,72	1014,54	6,06	0,244	9
Junho	"	88,08	790	39,19	0,642	12
Julho	"	88,60	844	2,46	0,027	10
Agôsto	"	88,10	487,5	11,51	0,766	10
Setembro	"	88,90	397,5	4,84	0,093	10



$\overline{X}$ — Pêso testículo.

b' — Pêso testículo/comprimento.

Fig. 1 — Variação do valor médio do pêso testicular e do coeficiente de regressão durante os meses do ano.

2. Constata-se com algumas exceções (Outubro de 1964 e Junho de 1965) que o coeficiente de regressão (b') é mais elevado nos meses de espermatogênese ativa que nos meses de repouso. Isto pode ser expresso da forma seguinte: o aumento ponderal do testículo é maior nos meses de atividade sexual que nos meses de repouso sexual, para um aumento unitário de comprimento do corpo (idade).

Devemos, aqui, considerar a significação "biológica" do coeficiente de regressão: em geral êste coeficiente mostra a variabilidade do pêso do testículo em relação ao crescimento corporal (idade). Nos meses de repouso sexual a variação é, em geral, relativamente pequena, mas no período da maturidade sexual, intervém um fator novo, que altera completamente esta regressão, aumentando enormemente a variação de pêso dos testículos, em função do comprimento do corpo. Embora a análise histológica do material não esteja ainda completa, podemos pensar que êste novo fator de variabilidade do pêso testicular seja o crescimento do epitélio germinativo com a onda de espermatogênese que ocorre, no período de atividade sexual. Não excluímos, igualmente outros fatôres concomitantes, como o acúmulo de sêmen e o fenômeno de embebição que podem acompanhar a maturidade espermatogenética.

Não comentamos por enquanto, a variação do coeficiente de correlação (r) que é extremamente variável durante os meses do ano por serem as amostras mensais constituídas de indivíduos de diferentes procedências e, como já dissemos a propósito da situação da espermatogênese, altamente variável entre os indivíduos de um mesmo mês.

Um exame superficial da situação da espermatogênese, nos diferentes indivíduos, durante vários meses, indica que embora haja uma notável variabilidade de indivíduo para indivíduo, as serpentes colhidas, nos meses de Janeiro a Maio mostram plena atividade espermatogenética, que está em declínio nos outros meses do ano. Porém, nos meses de inatividade, alguns dos testículos colhidos apresentavam atividade espermatogenética. Esta variabilidade de situação histológica do testículo de indivíduos colhidos, no mesmo mês, poderia ser explicada, eventualmente, pela diferente procedência das serpentes, pois a área de colheita do Instituto Butantan cobre climas muito diversos.

Aqui, apresentamos duas microfotografias (Figs. 2 e 3) de testículos, respectivamente em repouso e em atividade espermatogenética. O estudo sistemático do processo espermatogenético na cascavel, nos moldes da descrição das etapas feitas nos mamíferos pela Escola de Leblon e Clermont e por Roosen-Runge, está em andamento. A aplicação dêstes princípios aos répteis com períodos anuais de variação de atividade espermatogenética exige um estudo mais detalhado.

Podemos salientar, como conclusão geral, que êste estudo difere dos trabalhos executados pelos autores precedentes, que levaram em conta sòmente as variações estacionais do testículo. As pesquisas presentes combinam o estudo da variação do testículo com a idade do indivíduo e com a maturidade sexual. Êste modo de apreciar o problema nos permitiu averiguar a existência de dois fatôres de correlação: um primeiro fator que aparece, na regressão dos meses inativos e que corresponde ao crescimento anual do testículo em relação à idade e um segundo fator, que se sobrepõe nos meses ativos e que altera totalmente esta regressão básica.

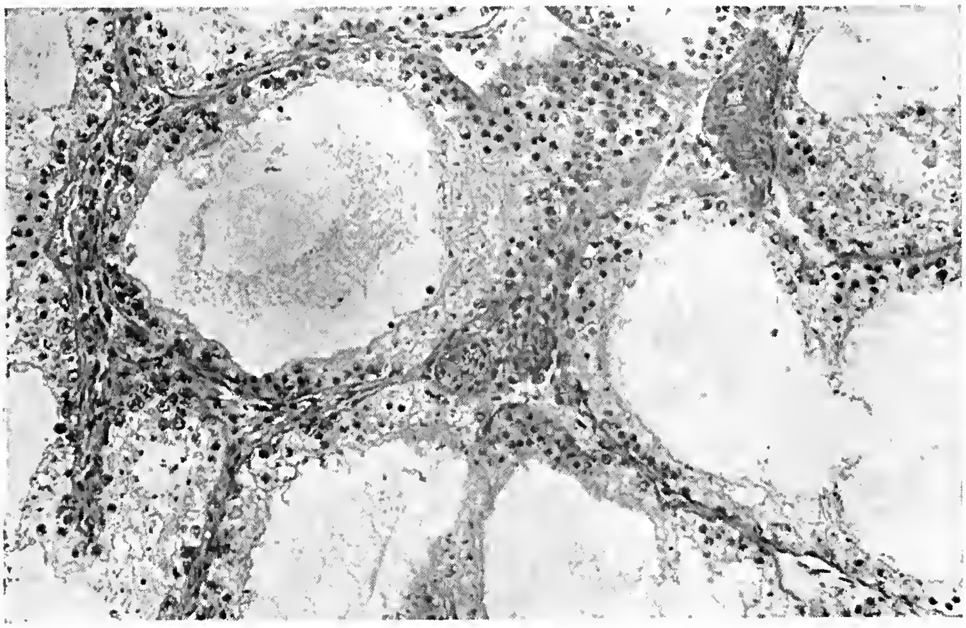


Fig. 2 — Testiculo de cascavel em repouso espermatogenético.

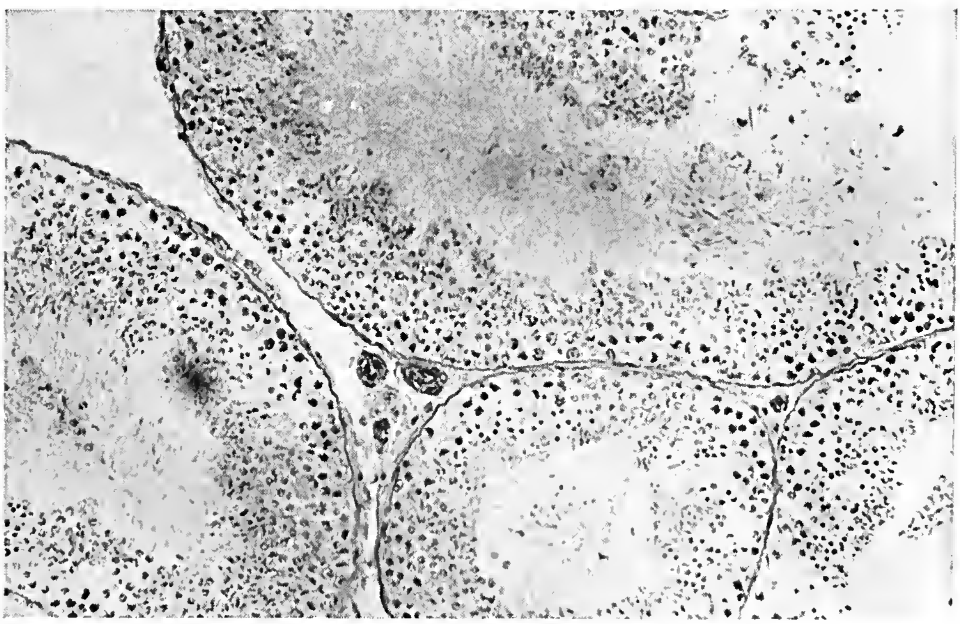


Fig. 3 — Testiculo de cascavel em plena atividade espermatogenética.

Summary

The cytological and ponderal variations of the testicle of the rattlesnake, *Crotalus durissus terrificus* (Laurentius) taken from the Butantan Institute during the year, have been studied from September, 1964 to September, 1965. Each month, about 10 individuals were studied. The regression line between the total length (age) and the testicle weight was calculated.

The weight of the testicle increases from September to April and decreases from then onward. In the same way, the regression coefficient increases from September to April and decreases during the inactive months.

The maximum of spermatogenetic activity is found during the period from January to May.

Some considerations are made concerning the biological significance of these variations of the regression coefficient.

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13. PROBABLE SIGNIFICANCE OF VENOM YIELD RECORD STUDIES

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The venom yield records are necessary for sera producing laboratories to know the output and numbers required for immunization. They are useful to the clinician to know the maximum amount of venom given by a snake so that in the case of a snake bite he is prepared for neutralizing that much amount of venom. It is also necessary for a biochemist to know the periodicity during the year and the toxicity thereby, to plan experiments for enzymes.

In the production of venom by snakes some of the factors that are supposed to affect the yield are, environment, sex, seasonality and frequency of milking; provided there is uniformity of feeding and age.

This Institute has been working on these aspects for some years and Deoras (1) gave the description of a snake farm wherein *Naja naja*, *Vipera russelli*, and *Bungarus coeruleus* were kept, after giving them a habitat which was seen for these snakes in nature. The snakes were milked for venom from the snake farm and this yield after lyophilization was compared to similar yield by the same snakes kept in cages in the room. The results of the comparison of these venom yield records have been given in the same paper and in the proceedings of the symposium held at the Pan Pacific Science Congress (2). In these comparative studies specific attention was given to taking different number of samples and sexes separately. The results of these individual studies showed that the output of venom in the farm by a cobra and Krait snakes both for male and female sexes was more than in rooms. This was not the case with the viper as is shown by table below which indicates significance or otherwise

TABLE I — STATISTICAL ANALYSIS OF AVERAGE VENOM PRODUCTION DATA TO SHOW WHETHER THE ENVIRONMENTAL DIFFERENCE IS SIGNIFICANT OR NOT

Year of Production	M/F	Cobra (*Naja naja*)	M/F	R. Viper (*Vipera russelli*)	M/F	Krait (*Bungarus coeruleus*)
1953	Vf-Vr	Significant		Not significant	Vf-Vr	Significant
1954	Vf-Vr	Significant		Not significant		Not significant
1955	Vf-Vr	Significant	Vr-Vf	Significante		Not significant
1956	Vf-Vr	Significant		Not significant	Vf-Vr	Significant

TABLE II — STATISTICAL ANALYSIS OF AVERAGE VENOM PRODUCTION DATA TO SHOW, WHETHER THE SEX DIFFERENCE IS SIGNIICANT OR NOT

Year of Production	M/F	Cobra (*Naja naja*)	M/F	R. Viper (*Vipera russelli*)	M/F	Krait (*Bungarus coeruleus*)
1955	Vm-Vf	Significant		Not significant	Vm-Vf	Significant
1957	Vm-Vf	Significant		Not significant	Vm-Vf	Significant
1958	Vm-Vf	Significant		Not significant	Vm-Vf	Significant

Vf = Venom of farm snake M/F = Vm verses Vf
Vr = Venom of room snake
Vm = Venom of male snake
Vf = Venom of female snake

The actual figures appear at Table No. V and VI of reference No. 2 quoted in this paper.

only; the figures are not given. A periodicity in milking and the production of venom output during different months as well as differential output of venom from different fangs especially by the Russell's viper was established (2). Since then improvements have been done in the keeping of snakes in the rooms and milking has been done in groups of both male and female snakes of different lengths together as will normally be practical for any laboratory. In the routine working of a laboratory, routine work cannot always be done with statistical data. It was therefore necessary to see if the results obtained in previous studies with controlled conditions are available in routine working. Vipers yield better in rooms. The caging conditions were improved as such it was necessary to see if the yield was still maintained. The present studies give an account of these subsequent observations and their probable significance.

Materials and Methods

The snakes in the rooms were kept in uniform size of tin cages. These had on two sides, a 16 mesh wire gauze and a 2 inch tall partition inside the cage, dividing it into two chambers. One that is below the cover, has a drinking water in a brass pot (clamped) and a number of rough stones. The other side has a small platform to allow the snake to rest on a dry surface. The stones are for rubbing the body during casting. The details about the rearing have been mentioned in the proceedings of the symposium on Animal husbandry in International Congress of Laboratory Animals, Dublin 1965. The room 46′ × 26′ contained 300 snake cages on racks. It had two exhaust fans running 12 hours during the night when doors and windows were closed. The maximum and minimum temperatures in room and farm were 31°C, 25.8°C, 31°C, and 24.9°C, respectively. The maximum and minimum relative humidity during the year in the room and the farm was 81%, 64%, 82%, and 67% respectively.

Total number of times that snakes were milked for these studies are 7960.

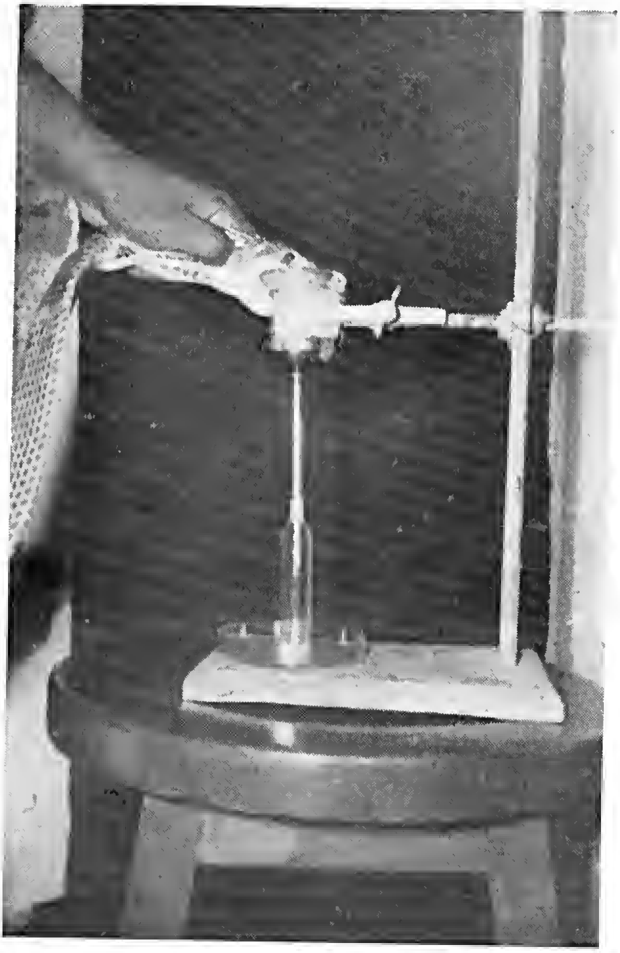

Fig. 1 — *Naja naja* biting a plastic coverstretched over a stainless steel funnel (whose stem leads into an ampoule for collection of venom).

Observations

Having seen significance in the output of venom by taking a group of snakes and even individual snakes of different sexes, we have now seen this in a large mixture group as will normally be available in any laboratory. Table III below, gives the observations from 1961 to 1965 wherein both male and female snakes have been taken together from farm and room. The results again shown as in the past work statistically analysed, that the output of venom given out by cobra and krait snakes is significantly more in the farm than the room. This is not the case with Russell's viper.

The periodicity of milking in the case of these snakes was one month. Table III gives the maximum and minimum output of venom for the different snakes. Table IV gives the entire yearly data showing the periodicity of output of venom in the room and farm in these snakes. The maximum outputs being concentrated during April to September months.

Individual snakes of different length and sexes were taken and milked in farm and room separately. Table V gives the data in detail for Krait and

TABLE III — SHOWING THE AVERAGE OUTPUT OF VENOM IN GMS AFTER LYOPHILIZATION, OF DIFFERENT SNAKES KEPT IN THE ROOM AND THE FARM FOR BOTH THE SEXES, AS WELL AS THE MAXIMUM & MINIMUM YIELDS

Kind of snake	Year	Total No. milked in 12 months.		Average venom output		Maximum venom output		Minimum venom output	
		Room	Farm	Room	Farm	Room	Farm	Room	Farm
	1961	238	332	0.0066	0.0307	0.0100	0.0385	0.0028	0.0240
	1962	308	216	0.0078	0.0208	0.0131	0.0337	0.0044	0.0173
Krait (*Bungarus coeruleus*)	1963	584	444	0.0103	0.0181	0.0153	0.0267	0.0071	0.0120
	1964	752	286	0.0082	0.0138	0.0136	0.0210	0.0058	0.0109
	1965	1.059	381	0.0090	0.0116	0.0115	0.0325	0.0044	0.0033
	1961	294	98	0.1081	0.1023	0.1700	0.1224	0.0570	0.0790
	1962	266	91	0.1357	0.1164	0.1729	0.1518	0.1189	0.0979
Russells viper (*Vipera russelli*)	1963	406	175	0.1514	0.1003	0.2000	0.1500	0.1060	0.0310
	1964	260	97	0.0851	0.0923	0.1290	0.1556	0.0651	0.0622
	1965	563	66	0.0725	0.0925	0.1640	0.1200	0.0490	0.0489
	1962	39	10	0.1807	0.2660	0.2012	0.3616	0.1692	0.1225
Cobra (*Naja naja*)	1964	105	20	0.1758	0.1515	0.2950	0.1866	0.0835	0.1221
	1965	657	223	0.1131	0.1272	0.1805	0.2136	0.0754	0.0275

TABLE IV — TABLE SHOWING THE AVERAGE OUTPUT AND PERIODICITY OF VENOM YIELD IN DIFFERENT SNAKES. (VENOM OF ONLY 13 SNAKES IN ONE AMPOULE).

1954	Cobra (*Naja naja*)		Viper (*Vipera russelli*)		Krait (*Bungarus coeruleus*)	
	Farm	Room	Farm	Room	Farm	Room
January	0.1480	0.0933	0.1110	0.0650	0.0167	0.0414
February	0.1710	0.1240	0.1310	0.0770	0.0121	0.0200
March	0.1810	0.1120	0.1290	0.1040	0.0250	0.0180
April	0.1810	0.1250	0.1310	0.1130	0.0290	0.0290
May	0.1810	0.1180	0.1670	0.1600	0.0280	0.0220
June	0.1700	0.1400	0.1670	0.1050	0.0275	0.0192
July	0.1390	0.1280	0.1250	0.0890	0.0240	0.0180
August	0.1327	0.0926	0.1760	0.0605	0.0354	0.0180
September	0.1589	0.1220	0.1564	0.1346	0.0360	0.0240
October	0.1235	0.0904	0.1237	0.1187	0.0260	0.0207
November	0.1714	0.0966	0.1280	0.1067	0.0210	0.0160
December	0.1193	0.1497	0.1062	0.1343	0.0314	0.0126
Average	0.1564	0.1160	0.1376	0.1015	0,0261	0.0216

average for Russell's viper and cobra indicating that the sexes and length inspite of being different, the results show that the output of venom in farm is better than in room for Krait and cobra and not significant fo the Russell's viper. It may here be noticed that beyond certain length, there is not much difference in the output of venom and that similar sizes may give more venom than bigger ones.

Venom collected from snakes was centrifuged and the supernatant liquid as well as the debris lyophilised. There was no or extremely small debris left in the case of cobra and krait snakes, but Russell's vipers gave a lot of debris. Table VI below shows the amount of venom in volume before centrifuging and the toxicity of the supernatant as well as the debris part. The table indicates that there is debris in vipers and the amount of debris left over may be only a dead bulk which is not toxic. Venom used in horses for immunization recently has shown that the present purified venom where debris has been discarded does not give the sloughing and other side reactions in horses as was available when this had not been removed.

Discussion

Stadelman (3), Fairley and Splatt (4), Amaral (5), and Schoettler (6) have recorded the differences in the output of venom in vipers of different locations. Kochwa (7) has tried to study the yield of venom in *Vipera palestinae*, but he has not made any attempts to keep snakes under natural surroundings. These

TABLE V — SHOWING THE PERIODICITY IN VENOM PRODUCTION DURING DIFFERENT MONTHS. WEIGHT OF VENOM IN GM AFTER LYOPHILIZATION

Krait (*Bungarus coeruleus*)

Months 1955	ROOM				FARM			
	Male length	Wt. of venom	Female length	Wt. of venom	Male length	Wt. of venom	Female length	Wt. of venom
January	4' 10"	0.0368	3' 11"	0.0012	4' 8"	0.0550	3' 4"	0.0050
February	4 1	0.0305	3 1	0.0006	4 11	0.0746	3 3	0.0086
March	4 9	0.0210	3 1	0.0044	4 0	0.0179	3 10	0.0135
April	3 9	0.0113	3 5	0.0067	5 0	0.0697	3 6	0.0097
May	4 5	0.0165	3 2	0.0092	5 0	0.0722	3 8	0.0832
June	4 8	0.0226	3 2	0.0028	4 4	0.0403	3 7	0.0223
July	3 8	0.0168	3 1	0.0176	4 9	0.0324	3 4	0.0166
August	4 2	0.0072	3 0	0.0104	4 1	0.0326	3 3	0.0148
September	4 11	0.0172	2 10	0.0064	4 8	0.0426	3 9	0.0159
October	4 4	0.0332	3 1	0.0112	3 8	0.0765	3 7	0.0218
November	3 1	0.0132	3 1	0.0092	3 11	0.0950	3 10	0.0319
December	3 9	0.0082	3 8	0.0098	3 10	0.0218	3 8	0.0419
Average	4' 3"	0.0195	3' 7"	0.0075	4' 5"	0.0525	3' 7"	0.0238
Russell's viper (*Vipera russelli*) Average venom yield in gm in 1955.								
Average	3' 10"	0.0952	3' 7"	0.0938	3' 9"	0.2072	3' 9"	0.2114
Cobra (*Naja naja*) Average venom yield in gm in 1955.								
Average	3' 10"	0.1145	3' 11"	0.1015	4' 6"	0.3003	4' 9"	0.1920

TABLE VI — SHOWING THE WEIGHT IN GMS OF LYOPHILIZED SUPERNATANT LIQUID AND DEBRIS IN VENOM UNDER PURIFICATION

Snake	No. snakes milked	Volume of Venom	Weight of Venom	Weights after centrifugation and Lyophilizing			
				Wt. of super-natant	Wt. of debris	M. L. D. of super-natant	M. L. D. of debris
Naja naja (Male)	5	3.0 cc.	2.8307	1.0230	Nil	1:120000	No debris
Naja naja (Female)	5	2.0 cc.	1.9291	0.7059	Nil	1:135000	Do.
Vipera russelli (Male)	5	5.5 cc.	5.1790	0.5371	0.0104	1:140000	Not toxic even at 1:10
Vipera russelli (Female)	5	4.7 cc.	5.0679	0.8553	0.0149	1:130000	Not toxic even at 1:10

surroundings were therefore made after studying the natural habitat and the results of experiments published in 1961 and 1963 (1, 2). These observations needed confirmation under routine conditions and data established for taking on over-all view about the keeping of snakes in the laboratory. There are a number of Institutions which are keeping snakes for immunological studies or for venom in connection with biochemical studies. Many have a structure called snake farms, in which the snakes are kept and exhibited. Some expenses are involved in keeping and exhibiting them. Our present studies have shown that the production of venom from cobra and Krait snakes is more from snakes kept in the farm where natural habitat is given than those kept in the room. Both these snakes have neurotoxic poison. The vipers which have haemotoxic venoms give more venom in the rooms. It has also been observed by us that the mortality of vipers kept in the farm is more than those kept in rooms as compared to cobra and krait snakes. It is therefore argued that if a snake farm is to be maintained in an institution for the production of venom, then it should have the natural surroundings of the habitat of that snake. Secondly the snakes like cobra and krait may be reared in such a kind of farm because there is more production of venom. For Russell's viper, the production is more in the room and the conditions there be improved to prevent the infection of viper throats by giving them cages where living and feeding compartments are separate. Laboratories working on immunological aspects should have venom production data for the year to plan immunological programmes for keeping the required number of snakes. It will be worthwhile experimenting whether the period when maximum amount of venom is produced is also the time to get most toxic venom. If so then the maximum collection be done during this time and venom used for antigenicity. The same could be available for biochemical studies. The figures for the maximum and average venom output for snakes give an indication to clinicians as to how much venom to expect in the worst cases when that particular snake bites. Lastly venom used for immunization be better centrifuged and supernatant liquid only used for the purpose.

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14. CONSTITUIÇÃO CROMOSSÔMICA E MECANISMO DE DETERMINAÇÃO DO SEXO EM OFÍDIOS SUL-AMERICANOS. II. CROMOSSOMOS SEXUAIS E EVOLUÇÃO DO CARIÓTIPO.

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O estudo cariotípico das serpentes cujos resultados já foram apresentados (1), serviu de base para a elaboração do presente trabalho.

Especial ênfase foi dada ao problema dos cromossomos sexuais visando esclarecer êsse aspecto ainda pouco conhecido nos répteis. Os resultados relativos à constância da massa cromossômica e à proporção do cromossomo Z em relação ao lote cromossômico são de interêsse, principalmente quando comparados à observações semelhantes feitas em outros grupos animais. Os microcromossomos, cuja natureza cromossômica nas aves foi negada por alguns autores, são discutidos em um tópico separado demonstrando-se que êsses elementos são cromossomos característicos, que se diferenciam dos macrocromossomos só pelo tamanho reduzido. Finalmente, quanto à evolução, são correlacionadas as observações cariotípicas, sendo sugerido um esquema de evolução para as serpentes estudadas.

Cromossomos sexuais

O padrão de herança típico dos genes ligados ao sexo está relacionado, freqüentemente, com um heteromorfismo do par de cromossomos portadores dêsses fatôres. Essa diferenciação morfológica dos heterocromossomos permite correlacionar aspectos citológicos e distribuição gênica, e tem sido uma das bases da teoria cromossômica da hereditariedade. No entanto, em numerosos casos de vertebrados, a demonstração da existência de uma heterogametia quanto aos cromossomos não foi até agora possível.

Nos peixes, o estudo de herança ligada ao sexo revela a existência de dois tipos de digametia. Gordon (2, 3), demonstrou que os peixes *Platypoecilus maculatus* "selvagens" do México têm um mecanismo genético de determinação do sexo (XX = fêmea; XY = macho) oposto ao encontrado nas criações de aquário da mesma espécie (ZW = fêmea; ZZ = macho). Gordon (4) verificou que o *Platypoecilus* das Honduras Britânicas pertence ao último tipo (ZW, ZZ). Castle, em 1936 (5), sugeriu que ZW e ZZ poderiam ser expressos como YX e YY respectivamente. Segundo Gordon (3) apesar do W dos peixes "domesticados" não ser homólogo ao X dos "selvagens", o Z e o Y eram aparentemente homólogos. Portanto, existiriam populações de *Platypoecilus* (com fêmeas heterogaméticas) nas quais, como condição normal, os machos seriam ZZ (YY). Yamamoto (6, 7) por sua vez verificou que no peixe *Oryzias latipes*, ocorre heterogametia masculina (XY), aparecendo excepcionalmente tam-

bém machos YY viáveis. A ocorrência de fêmeas WW em casos de heterogametia feminina também foi relatada. Belammy e Queal (8) criando o peixe "domesticado" *Platypoecilus maculatus*, encontraram vários machos ZW, o que confirma as observações anteriores de Breider (9). Verificaram que êsses machos quando cruzados com fêmeas normais ZW, davam origem a fêmeas WW, além das fêmeas ZW e machos ZZ normais. Progênies dessas fêmeas WW cruzadas com machos normais ZZ consistiram sòmente de fêmeas.

Nos anfíbios, sob o ponto de vista genético, ocorrem dois tipos de digametia, podendo esta variar dentro do mesmo gênero (10). Humphrey (11), demonstrou na salamandra *Amblystoma mexicanum* que a fêmea é genèticamente digamética. Quando fêmeas experimentalmente transformadas em machos eram cruzadas com fêmeas, a proporção na descendência era de três fêmeas para um macho. Os descendentes WW dêsse cruzamento (um têrço das fêmeas) eram fêmeas viáveis, pois, ao serem cruzadas com machos normais ZZ, produziram sòmente progênie feminina. Humphrey (12), utilizando a mesma espécie, conseguiu a reversão do sexo de fêmeas WW, através de implantação experimental de preprimórdio de gônadas de embriões. Essas observações em peixes e anfíbios sugerem que nas espécies de vertebrados inferiores os cromossomos sexuais encontram-se ainda em estágio tal de diferenciação que o X ou Z pode ser substituído pelo Y ou W, respectivamente.

Nas aves, o estudo experimental indicou a existência de uma digametia feminina. Yao e Olsen (13) observaram, por partenogênese, no peru, embriões exclusivamente masculinos o que seria explicado, segundo Poole e Olsen (14), admitindo uma digametia feminina; os ovócitos que retivessem o cromossomo Z na maturação dariam nascimento, após desdobramento do complemento haplóide, a indivíduos ZZ, portanto a machos, enquanto que os embriões OO (ou WW), resultantes de ovos onde o cromossomo Z passou ao glóbulo polar, morreriam.

Nenhum dêsses métodos foi utilizado para elucidar o tipo de digametia entre répteis; sòmente Dantchakoff (15) tentou relacionar os resultados de suas experiências endocrinológicas em mamíferos, aves e répteis com o tipo de digametia que ela presumia feminina nesses últimos.

Quanto aos estudos citológicos, Nogusa (16) descreveu no peixe *Mogrunda obscura* um par de cromossomos XY no macho, cujo heteromorfismo poderia ser observado na metáfase I. Êsse heteromorfismo não é, no entanto, convincente, segundo van Brink (17).

Nos anfíbios, Witschi (18) e Yosida (19) descreveram respectivamente em *Rana temporaria* e *Hyla arborea* dimorfismo dos cromossomos sexuais no macho, mas também, as diferenças encontradas foram extremamente pequenas. Já no *Xenopus laevis*, que é um dos anuros mais primitivos, no qual a heterogametia feminina havia sido provada experimentalmente por transformação do sexo, Chang e Witschi (20), Gallien (21) e Weiler e Ohno (22) identificaram citològicamente um grande dimorfismo dos cromossomos sexuais, na fêmea. O cromossomo Z é o menor elemento do cariótipo, enquanto que o W excede em tamanho o maior autossomo. Segundo êsses autores, em algumas espécies de vertebrados inferiores, e êste seria o caso de *Xenopus laevis*, a diferenciação morfológica dos elementos sexuais pode ter precedido sua diferenciação genética real.

Nas aves, em *Gallus domesticus*, a primeira confirmação citológica da heterogametia feminina deve-se a Suzuki (23), que identificou o quinto elemento, em ordem de tamanho como sendo o cromossomo Z. Subseqüentemente vários investigadores confirmaram êsses achados. Miller (24), estudando uma galinha com transformação de sexo, demonstrou que o cromossomo Z, ímpar no sexo

heterogamético, passa a sòmente um pólo do fuso, durante a primeira divisão da meiose. Posteriormente verificou que o Z não é pareado com qualquer dos cromossomos menores na placa equatorial, favorecendo assim a hipótese da constituição ZO e não ZW no sexo heterogamético. Oguma (25) fixou em 78, o número diplóide para o macho e 77 para a fêmea de *Gallus domesticus*, admitindo, também, a constituição cromossômica ZO para o sexo heterogamético. Yamashina (26) numa série de trabalhos descreveu condições análogas, num grande número de espécies estudadas. Ohno (27) estudando células meióticas e mitóticas de *Gallus domesticus*, encontrou também só um Z na fêmea heterogamética. Segundo êle, o número de cromossomos é pouco acima de 70, mas admite, como o fizeram Matthey e van Brink (28), que a determinação exata do número de cromossomos nessa espécie é ainda impossível. Segundo van Brink (17), das 29 espécies de aves estudadas por vários autores, 18, pertencentes a 8 famílias diferentes, apresentam digametia feminina reconhecível ao nível cromossômico. Nas 11 espécies restantes é impossível, à base dos dados existentes, um pronunciamento seguro.

Oguma, em 1934 (29), relatou o primeiro caso de digametia feminina nos répteis, constatando que o macho de *Lacerta vivipara* possui 36 e a fêmea 35 cromossomos, admitindo assim a existência de uma digametia ZO também entre os répteis. Em 1937 (30) encontrou o mesmo tipo de digametia na tartaruga *Amyda japonica* (macho = 64; fêmea = 63). Makino e Asana (31) descreveram o mesmo fenômeno em *Calotes versicolor* (macho = 34; fêmea = 33) e em *Sitana ponticeriana* (macho = 46; fêmea = 45). Nakamura (32) confirmou a existência do mecanismo ZO na fêmea da tartaruga *Caretta caretta olivacea* (2n = 57) e na *Chelonia japonica*, Makino (33) encontrou 56 elementos no macho e 55 na fêmea. Nesse mesmo trabalho, relata ter reexaminado as preparações de Oguma confirmando as conclusões dêsse autor. Também Suzuki (34) relata cromossomos sexuais do tipo ZO-ZZ em *Amyda maacki*.

Por outro lado Matthey (35) não observou nenhuma diferença cromossômica nos dois sexos de *Chamaeleon vulgaris*, que possui 2n = 24 cromossomos. Margot (36) também não encontrou diferenças cromossômicas nos dois sexos de *Anguis fragilis* (2n = 44) e *Lacerta vivipara* (2n = 36). Num outro sáurio *Chamaeleon bitaeniatus*, cuja análise cromossômica é muito fácil pelo pequeno número de cromossomos (2n = 24) e por ter apenas 4 microcromossomos, Matthey e van Brink (37) também não observaram digametia. Em 1957, Matthey (38) relatou a ausência de digametia feminina em outros CHAMAELEONTIDAE: *Brookesia stumpfii* (2n = 24), *Chamaeleon campani* (2n = 26), *Chamaeleon brevicornis* (2n = 32) e *Chamaeleon nasutus* (2n = 34). Êsses estudos levaram-no à convicção da inexistência generalizada de cromossomos sexuais morfològicamente diferentes nos répteis. Em 1962, Kobel (39) relata ter encontrado um par dimórfico de cromossomos sexuais numa fêmea estudada de *Vipera berus*.

A raridade de observações de digametia feminina é provàvelmente devida, em parte, às dificuldades concernentes aos métodos de estudo até há pouco utilizados. Para evidenciar os heterocromossomos os autores têm utilizado, geralmente, técnicas que envolvem o estudo da meiose. A digametia seria identificada pela presença de um bivalente assimétrico nos estados meióticos que precedem a anáfase I e pelo comportamento eventualmente aberrante, alocíclico, dêste bivalente durante a prófase da meiose, assim como durante a anáfase I. Ora, as células em meiose são geralmente abundantes nos testículos do macho, onde um heteromorfismo morfológico é fàcilmente observável. Na fêmea, porém, as divi-

sões meióticas, além de serem menos numerosas que no macho, ocorrem num período onde o ôvo se encontra repleto de substâncias de reserva, o que dificulta uma fixação perfeita do material. Portanto, o melhor meio para se investigar a presença de um par heteromorfo na fêmea é a análise numérica e morfológica dos cromossomos na mitose; no caso de digametia do tipo ZZ-ZW encontraremos números pares em ambos os sexos, mas na fêmea um dos pares será constituído de elementos desiguais.

Nossas observações nos ofídios indicam que as espécies não venenosas estudadas, da família BOIDAE, não apresentam cromossomos sexuais morfològicamente diferentes que permitam sua distinção em relação ao restante do cariótipo. Essa ausência de diferenciação morfológica não indica a inexistência de cromossomos sexuais, pois, através do processo evolutivo, podem, nessas espécies, ter-se acumulado genes masculinizantes e feminilizantes em um ou mais pares de cromossomos, que condicionariam a determinação do sexo.

As espécies incluídas nas famílias COLUBRIDAE e CROTALIDAE, por outro lado, caracterizam-se por apresentarem no cariótipo um par de heterocromossomos, que foi por nós identificado como sendo o quarto par de macrocromossomos em ordem de tamanho. Os machos constituem o sexo homogamético apresentando, no quarto par, dois cromossomos metacêntricos idênticos, que corresponderiam aos cromossomos ZZ, ou XX dos outros animais. As fêmeas por sua vez constituem o sexo heterogamético apresentando, no quarto par, dois cromossomos morfològicamente diferentes, sendo um semelhante ao cromossomo metacêntrico Z ou X encontrado no macho, enquanto que, o outro cromossomo é menor, acrocêntrico e corresponderia ao cromossomo W ou Y dos outros animais.

Nos COLUBRIDAE e CROTALIDAE, o mecanismo cromossômico de determinação do sexo é mais diferenciado do que nos BOIDAE. Além do acúmulo diferencial de genes, já teria ocorrido uma diferenciação morfológica entre os cromossomos sexuais. Essa hipótese, que supõe um mecanismo cromossômico de determinação do sexo mais diferenciado nos COLUBRIDAE e CROTALIDAE, estaria em perfeito acôrdo com a posição sistemática ocupada pelas espécies estudadas, pois, não resta dúvida que, por exemplo, as espécies peçonhentas representam um grupo mais evoluído, em relação as espécies que não são venenosas. A simples presença de um aparelho inoculador de veneno altamente especializado nas serpentes peçonhentas, caracteriza o seu grau de evolução em relação as outras espécies, dentre as quais algumas ainda apresentam vestígios remanescentes da sua evolução a partir de grupos sistemàticamente mais primitivos. Por exemplo, nos BOIDAE é ainda evidente a presença de membros locomotores atrofiados.

Na família COLUBRIDAE estão incluídas as serpentes que apresentam as maiores variações de número e morfologia dos cromossomos, inclusive do par de cromossomos sexuais. Porisso mesmo constituem excelente material para estudo dos mecanismos de alterações cariotípicas nas serpentes. No que diz respeito aos cromossomos sexuais, que pretendemos discutir neste capítulo, verificamos que tôdas as espécies de colubrídeos que estudamos apresentam um par heteromórfico na fêmea. A sua morfologia e tamanho em relação aos outros cromossomos é, no entanto, variável.

Em *Spilotes pullatus anomalepis, Spilotes pullatus maculatus, Phylodrias olfersii olfersii, Dryadophis bifossatus bifossatus, Drymarchon corais corais* e *Chironius bicarinatus*, ambos os cromossomos sexuais têm aproximadamente o mesmo tamanho, diferindo sòmente pela posição do centrômero, que é terminal

no Z e submediano no W. Aparentemente, a quantidade de material genético é a mesma nos dois cromossomos. Baseando-nos no aspecto morfológico do par de heterocromossomos, poderíamos sugerir uma explicação para a diferenciação dêsses cromossomos sexuais. É possível que tenha ocorrido uma inversão pericêntrica num dos homólogos do quarto par de cromossomos. Em conseqüência dessa inversão, a permutação entre os homólogos ficaria reduzida consideràvelmente. Êsse mecanismo de isolamento, pode ter resultado num maior acúmulo diferencial de genes relacionados à determinação do sexo, nos cromossomos em questão.

O tipo de cromossomos sexuais apresentado, por exemplo, pela *Spilotes*, pode representar um grau intermediário na evolução dêsse par cromossômico em direção ao apresentado pelos crotalídeos. Nestes últimos, o tamanho relativamente menor do cromossomo W parece ser conseqüência de uma redução por eliminação de material cromossômico. É possível que a redução de tamanho do W tenha conduzido nos animais mais evoluídos como os mamíferos, ao diminuto cromossomo Y, que teria como função a de induzir o desenvolvimento das gônadas masculinas.

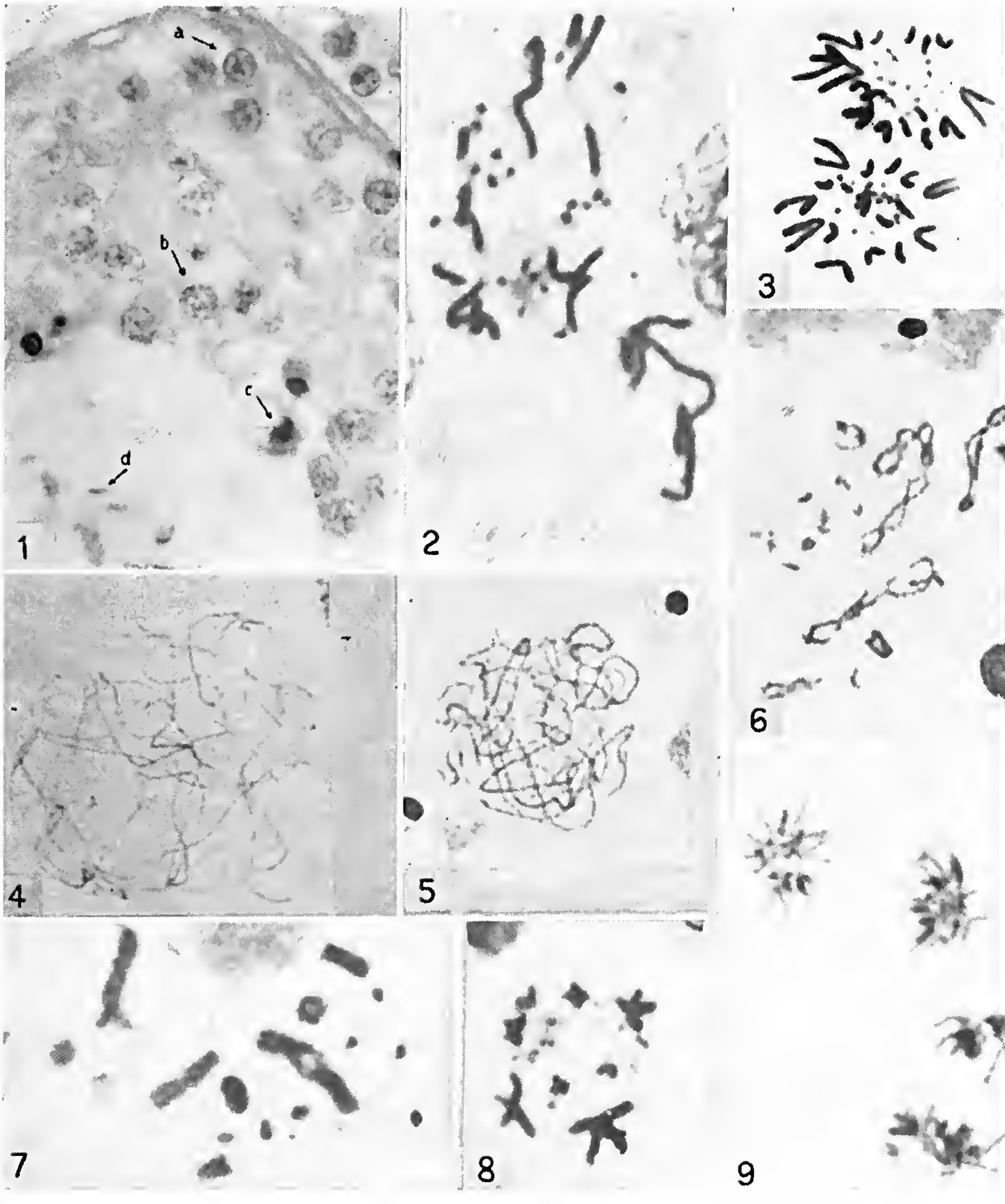
Em *Clelia occipitolutea*, o par de cromossomos sexuais é representado por um W, que é o maior cromossomo do cariótipo e por um Z, que ocupa o quarto lugar em ordem de tamanho em relação aos autossomos. Essa situação peculiar, na qual o cromossomo que ocorre só no sexo heterogamético é muito maior que o outro cromossomo sexual, foi descrita recentemente também num outro organismo, *Xenopus laevis* (22). O tamanho maior do cromossomo W pressupõe um acúmulo também maior de genes, que aparentemente não ocorreriam no sexo homogamético, que possui sòmente cromossomos Z.

A observação de Weiler e Ohno (22) sôbre a existência dêsse dimorfismo cromossômico acentuado num anuro tão primitivo como o *Xenopus laevis* poderia ser explicada, segundo êsses autores, como conseqüência de uma diferenciação morfológica dos cromossomos sexuais antecedendo a uma maior diferenciação genética real dos mesmos. Em outras espécies, apesar da impossibilidade de distinguir morfològicamente os cromossomos sexuais, pode na realidade existir um grande acúmulo diferencial de fatôres masculinizantes e feminilizantes. Poderíamos, também, com base nessas observações e nas nossas sugerir a hipótese de que a evolução quanto à diferenciação dos cromossomos sexuais não é sempre uniforme, no sentido de acompanhar a escala zoológica. Dependendo das condições em que se desenvolve determinado grupo animal, o seu mecanismo cromossômico de determinação do sexo pode ser mais diferenciado do que o de um grupo zoològicamente superior como é o caso, por exemplo, do anfíbio *Xenopus laevis* em relação às serpentes BOIDAE.

Espermatogênese

Para o estudo da espermatogênese nos ofídios, foi escolhida a espécie *Bothrops jararaca*, pela facilidade local de obtenção de material e porque nessa espécie o cariótipo tem 36 cromossomos, que é o mais comumente encontrado nas serpentes, além de que o heteromorfismo dos seus cromossomos sexuais é evidente (40).

O método utilizado foi o de esmagamento do testículo e coloração pelo Giemsa ou orceína acética (1). As preparações foram feitas no segundo semestre dos anos de 1962 e 1963, na época de reprodução dessas serpentes, que apresentaram então abundância de células em meiose (Fig. 1).



Figuras 1 a 9

Espermatogônias

Na gônada masculina, as espermatogônias apresentavam-se principalmente nos estágios de prófase e metáfase. Nas células em prófase os cromossomos encontram-se distendidos sob a forma de filamentos longos provàvelmente devido ao afrouxamento da espiral. Pôde-se, no entanto, distinguir fàcilmente a presença de 36 elementos, que consistem de 16 macrocromossomos e 20 microcromossomos.

Na prófase da gônia os cromossomos encontram-se distendidos e em certos cromossomos pode-se observar a associação com o nucléolo, através das assim chamadas regiões organizadoras do nucléolo. Nas espermatogônias de *Bothrops jararaca* tanto na prófase precoce como na prófase tardia verificamos que o nucléolo encontrava-se associado a 4 ou 5 microcromossomos (Fig. 2). Em algumas células encontrou-se um macrocromossomo próximo ao nucléolo, mas devido à inconsistência dêsse achado em outras células, êsse aspecto foi considerado como fortuito.

Devido ao tamanho reduzido dos microcromossomos de *Bothrops jararaca*, não foi possível com as técnicas utilizadas, identificá-los, no sentido de verificar se os que se encontram associados ao nucléolo pertencem a pares homólogos ou se são 4-5 microcromossomos pertencentes a pares diferentes.

Na cobaia, *Cavia cobaya*, foram observados um ou dois nucléolos nas prófases diplóides de ambos os sexos. Apesar de ambos os membros do par de autossomos de maior tamanho possuirem aparentemente capacidade inerente de organização do nucléolo, verificou-se que sòmente um dos homólogos apresenta-se associado a um nucléolo. É, portanto, possível que certas regiões de cromossomos homólogos da mesma célula não funcionem sincrônicamente (41).

Na galinha, *Gallus domesticus*, foi também observada a existência de um só nucléolo e êste apresenta-se associado aos microcromossomos, mas nesse organismo o número de microcromossomos associado foi avaliado em aproximadamente 12 (42).

Mais de 60 prófases somáticas e de espermatogonias examinadas, em várias preparações de testículo de *Bothrops jararaca*, não demonstraram a existência de qualquer macrocromossomo inteiramente heteropicnótico. Essa análise permite concluir que, tanto nas células somáticas como nas espermatogônias do macho dêsse ofídio, nenhum dos cromossomos sexuais apresenta heteropicnose positiva.

No testículo, as metáfases somáticas e das espermatogônias apresentam 36 elementos, sendo 16 macrocromossomos e 20 microcromossomos, como já descrevemos nas metáfases somáticas de culturas de leucócitos (40). Também nesse estágio nenhum dos cromossomos exibe heteropicnose positiva. Todos os macrocromossomos apresentam estrutura de duplas cromátides, que se apresentam conectadas sòmente pela região do centrômero. Os microcromossomos têm estrutura idêntica, apesar de que em alguns, devido ao tamanho reduzido, é difícil localizar-se a posição do centrômero.

Na anáfase os centrômeros, através dos quais as cromátides mantinham-se juntas, dividem-se, deslocando-se para pólos opostos da célula. Em conseqüência, as cromátides separam-se, afastando-se. Observa-se divisão equitativa do material cromossômico, isto é, tanto as cromátides que constituem os macrocromossomos como as que formam os microcromossomos separam-se igualmente migrando para os pólos (Fig. 3). Os microcromossomos por terem tamanho menor apresentam às vêzes as suas cromátides completamente separadas, antes dos macrocromossomos.

Na telófase os dois complementos cromossômicos, morfològicamente idênticos, separam-se completamente dando origem a dois novos núcleos diplóides.

Meiose

Nos espermatócitos de primeira ordem foi possível encontrar todos os estágios que caracterizam a prófase meiótica. A primeira evidência do início da meiose, numa fase que chamaremos de pré-leptóteno, é, em comparação com a célula em interfase, uma ligeira rarefação do material nuclear, com um princípio de delineamento dos filamentos cromossômicos. Na fase de leptóteno êsses filamentos tornam-se mais evidentes, mas ainda finos, apresentando pequena afinidade pelo corante.

Na fase de zigóteno, os filamentos homólogos atraem-se, ocorrendo o pareamento, simultâneamente, em vários pontos ao longo dos cromossomos e nas extremidades. É freqüente observar-se nessa fase células em que os filamentos encontram-se quase completamente pareados, exceto em alguns pontos intersticiais (Fig. 4).

Células em paquíteno são muito freqüentes. Nessa fase os cromossomos homólogos apresentam-se mais condensados e conjugados, formando bivalentes. Ao longo dêles observam-se, às vêzes, regiões mais condensadas, que correspondem aos cromômeros. Os microcromossomos apresentam-se, como o restante dos cromossomos, isolados, formando bivalentes, podendo-se, às vêzes, distinguir cromômeros nos mesmos. Ao contrário do que foi descrito por Newcomer e Brant (43) na galinha, os microcromossomos de ofídios não apresentam heteropicnose positiva, que os distinga dos macrocromossomos nesta ou em qualquer outra fase da meiose. Certas regiões dos macrocromossomos apresentam uma condensação mais pronunciada. Essas partes mais condensadas parecem resultar do maior acúmulo de heterocromatina e se localizam geralmente em regiões adjacentes aos centrômeros e nas extremidades dos cromossomos (Fig. 5).

Na fase de diplóteno, os cromossomos homólogos, que no estágio precedente apresentavam-se completamente conjugados, separam-se exceto nas regiões onde provàvelmente ocorrem quiasmas (Fig. 6). Êsse fenômeno pode ser claramente observado em todos os macrocromossomos. Apesar da dificuldade em determinar-se o número exato de quiasmas de cada bivalente, verificamos que o número de quiasmas no primeiro bivalente é o mais alto, sendo por nós avaliado em seis a oito aproximadamente por célula. No segundo e terceiro bivalente foram contados de quatro a seis quiasmas. No quarto bivalente, que corresponde em ordem de tamanho aos cromossomos sexuais, ocorrem de três a cinco quiasmas. Nos outros bivalentes de macrocromossomos observamos de dois a três quiasmas. Os microcromossomos também apresentam quiasmas, mas em número reduzido, sendo a sua avaliação mais difícil.

A fase de diacinese pouco se distingue do diplóteno, mas, pode ser caracterizada pela maior condensação dos bivalentes, que se coram mais intensamente que nas fases anteriores. Ocorre uma reduzida terminalização dos quiasmas, não se observando a formação de figuras em anel. Nessa fase, a avaliação do número de quiasmas é ainda possível, porém é mais difícil.

A existência de diplóteno e diacinese característicos e a ocorrência de freqüência relativamente alta de quiasmas em *Bothrops jararaca* mostram que, quanto à evolução genética, os ofídios são mais semelhantes às aves que aos anfíbios. Realmente, Weiler e Ohno (22), estudando preparações de testículo adulto do

anuro *Xenopus laevis*, não encontraram figuras de paquíteno e diplóteno precoce. Nesse animal, na meiose do espermatócito de primeira ordem, os bivalentes aparentemente passam do estágio de zigóteno ao de diplóteno tardio ou diacinese, apresentando nessa fase, todos os bivalentes forma de anel. Mesmo nos bivalentes grandes não foi observado quiasma intersticial. Essa observação está de acôrdo com o trabalho de Wickbom (44) sôbre a baixa freqüência de quiasmas nos anuros, que segundo Witschi (45), estaria correlacionada ao reduzido ritmo de evolução nesse grupo. Nas aves, por sua vez, não só ocorre paquíteno e diplóteno, como nas cobras, mas também o número de quiasmas é alto. Segundo Ohno (27), o primeiro e segundo pares de bivalentes apresentam de oito a dez quiasmas e o terceiro e quarto têm seis quiasmas. O quinto par, que corresponde aos cromossomos sexuais, apresenta forma de anel ou de 8 na diacinese, enquanto os bivalentes maiores apresentam vários quiasmas. Êsse fenômeno, no entanto, é devido à rápida terminalização do bivalente sexual, pois no diplóteno é possível observar que êsse bivalente tem de quatro a cinco quiasmas.

Por outro lado, a presença nos répteis e nas aves de microcromossomos, que não ocorrem aparentemente nos anfíbios, é um outro fato que indica a maior semelhança entre os dois primeiros grupos citados.

Na metáfase, os cromossomos apresentam-se uniformemente mais condensados. Os bivalentes dispõem-se na placa equatorial, com os centrômeros no equador. Cada cromossomo encontra-se dividido nesse estágio em duas cromátides, portanto, cada bivalente é constituído de quatro filamentos, podendo o conjunto ser denominado tétrade. Alguns dos bivalentes, principalmente os maiores, não se apresentam ainda completamente terminalizados (Fig. 7).

Na anáfase, os centrômeros homólogos dos bivalentes separam-se, deslocando-se para pólos opostos do fuso celular. Os microcromossomos separam-se como o restante dos bivalentes, eqüitativamente, recebendo cada célula filha, dez microcromossomos.

Na intercinese, os dois lotes de cromossomos encontram-se já completamente separados nos espermatócitos de segunda ordem. Os cromossomos, ou díades, aparecem mais distendidos, sob a forma de finas estruturas filamentosas.

Na prófase II da meiose, os cromossomos encontram-se mais separados, nas preparações. O número de elementos observados nessa fase é de 18, sendo 8 maiores que correspondem aos macrocromossomos e 10 menores que correspondem aos microcromossomos. Certas regiões dos cromossomos, principalmente na proximidade dos centrômeros, parecem mais marcadas por heterocromatina.

Na metáfase II, os cromossomos, ou díades, num total de 8 macrocromossomos e 10 microcromossomos aparecem fortemente condensados. Os braços são relativamente mais curtos e largos que na fase anterior. Em tôdas as figuras principalmente nos cromossomos maiores, observa-se que os braços estão bastante separados, formando pràticamente ângulos retos entre si (Fig. 8).

Na anáfase II, os centrômeros dividem-se e as duas cromátides de cada cromossomo separam-se, deslocando-se a polos opostos do fuso (Fig. 9).

Na telófase II, os cromossomos já se encontram completamente separados, dando origem a duas células haplóides com 18 cromossomos cada. Os filamentos começam novamente a alongar-se tornando-se aparentemente menos visíveis. É comum observar-se nas preparações a divisão sincrônica das células, que se originaram do mesmo espermatócito, resultando em quatro células haplóides, próximas e morfològicamente semelhantes.

Em nenhuma das fases acima descritas, quer na divisão das espermatogônias quer na primeira e segunda divisões meióticas, foi observado comportamento alocíclico de um ou de ambos os cromossomos sexuais. Aparentemente êstes comportam-se como o restante dos autossomos, durante a espermatogênese.

Cromatina sexual

A cromatina sexual, também conhecida por corpúsculo cromatínico ou de Barr, foi originalmente descrita por Barr e Bertram (46) em células nervosas de gatas, tendo sido subseqüentemente demonstrada também em células somáticas humanas e de outros mamíferos.

A cromatina sexual é característica das células femininas, onde se apresenta como uma massa basófila, Feulgen-positiva, na face interna da membrana nuclear.

A presença dessa estrutura nos núcleos femininos, era interpretada, até recentemente, como regiões heterocromáticas dos dois cromossomos X que na intérfase somática apresentar-se-iam condensadas e fortemente coráveis, isto é, heteropicnóticas. No entanto, na célula feminina em intérfase apenas um cromossomo X é heteropicnótico, sendo o outro indistinto dos autossomos.

Segundo Lyon (47), nas células somáticas femininas, sòmente um X seria genèticamente ativo durante a intérfase, apresentando-se o outro condensado. Numa fase precoce da embriogênese, provàvelmente ao tempo da implantação, em cada célula seria "decidido" qual dos dois cromossomos X seria ativo. Nas células masculinas normais os cromossomos X seriam presumìvelmente ativos. A linhagem germinativa da fêmea não participa, aparentemente, dêsse processo de diferenciação do cromossomo X. A hipótese de Lyon de diferenciação do X baseia-se citològicamente no fato de ter-se observado heteropicnose de um dos cromossomos X nas células femininas em prófase (48).

Por outro lado, empregando-se técnicas de autoradiografia em culturas de tecidos de hamster (49) e humanos (50), (51), (52) tratadas pela timidina tritiada verificou-se que um dos dois X da célula feminina, replica o seu ADN relativamente mais tarde que o outro X e os autossomos. Êsse cromossomo "tardio" não foi encontrado nas células masculinas.

Para investigar a existência da cromatina sexual nos ofídios foram feitas preparações de cérebro, baço, fígado, rim e gônadas. As lâminas foram preparadas pelas técnicas de esmagamento ou corte histológico, seguida de hidrólise e coloração pelo Giemsa ou Hematoxilina-Eosina. Nas preparações examinadas de *Boa constrictor amarali* e *Bothrops jararaca* não observamos em nenhum dos sexos a existência de uma massa condensada, que correspondesse à cromatina sexual. Em algumas preparações apareceram corpúsculos condensados nos núcleos de algumas células, mas êstes não se encontravam, geralmente, na periferia do núcleo e eram esparsos. Além disso, essa ocorrência foi observada indistintamente nos dois sexos.

Examinamos também preparações de baço e gônadas, obtidas por esmagamento, em outras ordens de répteis. Foram estudados *Anolis carolinensis* (**LACERTILIA**), *Caiman sclerops* (**CROCODILIA**) e *Amyda ferox* (**CHELONIA**). Nesses animais também em nenhum dos sexos foi observado um corpúsculo que pudesse ser considerado como cromatina sexual.

Em *Gallus domesticus*, Kosin e Ishizaki (53) relataram uma diferença nuclear entre o macho e a fêmea. Êsses autores identificaram um corpúsculo correspondente à cromatina sexual nos núcleos somáticos do sexo heterogamético. Encontraram também um corpúsculo nos núcleos de machos, mas em freqüência muito menor. Ohno, Kaplan e Kinosita (54) confirmaram a existência da cromatina sexual na fêmea de *Gallus domesticus*, demonstrando que a mesma representa o cromossomo Z, ímpar, em heteropicnose positiva. Por outro lado, Schmid (55) empregando timidina tritiada em cultura temporária de medula óssea, verificou, por autoradiografia, que os dois cromossomos Z do macho homogamético e o Z ímpar da fêmea heterogamética não apresentam diferenças entre si ou em relação aos autossomos, quanto à incorporação de timidina.

A condensação de um cromossomo X nas células somáticas femininas, nos mamíferos, parece ser um mecanismo muito especializado. Aparentemente a diferenciação dos cromossomos X e Y seria tal que a maior parte dos genes contidos no cromossomo X se encontraria em dose simples na célula diplóide masculina, sem correspondência no cromossomo Y. Paralelamente a êsse fenômeno, a condensação de um cromossomo X nas células femininas compensaria a situação de hemizigose do cromossomo X, nas células masculinas. Nos répteis, aparentemente, não se desenvolveu êsse tipo de mecanismo compensador.

Massa cromossômica e proporção Z : Autossomos

O estudo contínuo de material cromossômico em diferentes organismos despertou o nosso interêsse em encontrar algumas regras gerais que governassem a variedade de cariótipos exibidos pelos animais. Estudamos os cromossomos de sete espécies e um híbrido interespecífico de mamíferos placentários pertencentes às ordens **PRIMATA**, **ARTIODACTYLA**, **PERISSODACTYLA**, **CARNIVORA** e **RODENTIA**, cujo número cromossômico diplóide varia de 17/18 no *Microtus oregoni* a 78 no *Canis familiaris* (56). Através de um método que nos permitiu um cálculo bidimensional da área dos cromossomos, obtivemos resultados que nos levaram a conclusões interessantes.

Apesar da grande variação no número cromossômico, as oito espécies estudadas parecem conter quantidades semelhantes de material genético, que é de aproximadamente 155 μ^2. O cromossomo X da maior parte dessas espécies (*Homo sapiens*, *Canis familiaris*, *Bos taurus*, *Equus caballus*, *Equus asinus*, *Felis domestica*, *Mus musculus*), parece ter área quase idêntica e equivalente a aproximadamente 4,5 μ^2, que é um pouco mais de 5% do lote haplóide da espécie considerada. No *Mesocricetus auratus*, o X apresenta aproximadamente o dôbro de tamanho do X dessas espécies, sendo por nós considerado como duplo e no *Microtus oregoni* o X tem mais ou menos três vêzes o tamanho do X dessas espécies, sendo considerado como triplo. Estudando o comportamento heteropicnótico dos três tipos de cromossomos X nas células somáticas, verificamos que êstes apresentam padrões diferentes. Se admitirmos que a heteropicnose de um cromossomo ou parte dêle significa inércia genética então, apesar das diferenças de tamanho dos três tipos tôdas as espécies estudadas apresentam aparentemente a mesma quantidade de material cromossômico X funcional, em ambos os sexos.

Nos ofídios o cariótipo mais comumente descrito, incluindo o presente trabalho, é o que possui 36 cromossomos compreendendo 16 macrocromossomos e 20 microcromossomos. No entanto, cariótipos com número cromossômico diferente têm sido encontrado em várias espécies. Os valôres extremos encontrados são os das espécies *Xenodon merremii* e *Clelia occipitolutea*, presentemente des-

critos. Na primeira espécie ocorrem 30 e na última 50 cromossomos como número diplóide.

Com o intuito de verificar a significação dessa variação numérica quanto à massa total de material cromossômico e a relação do cromossomo sexual Z com o complemento cromossômico, resolvemos estudar êsses parâmetros, nos ofídios. Para possibilitar o estudo comparativo com as espécies de mamíferos pesquisadas usamos o mesmo método e ampliação que havíamos utilizado para o estudo anterior.

Selecionamos preparações citológicas de sete espécies de serpentes pertencentes a três famílias diferentes. Cinco dessas espécies apresentam cariótipo com 36 cromossomos, que é o número mais característico dos ofídios, e duas outras espécies apresentam a variação mínima e máxima do número de cromossomos nesse grupo.

Os CROTALIDAE são uma família muito homogênea, sem grandes variações, aparentemente. Tôdas as espécies que estudamos têm 36 cromossomos. Escolhemos as espécies *Bothrops jararaca* e *Bothrops atrox*. Os BOIDAE também são uma família homogênea, apresentando as espécies estudadas como número diplóide, 36 cromossomos. A única exceção encontrada nos boídeos, foi a espécie *Corallus caninus*, que tem 44 cromossomos. Escolhemos para êsse estudo as espécies *Boa constrictor amarali* e *Epicrates cenchria crassus*. Quanto aos colubrídeos a heterogeneidade é maior. Com o intuito de estudar a significação da variação numérica dos cromossomos, escolhemos para essa investigação três espécies, *Spilotes pullatus anomalepis*, que tem como número diplóide 36 cromossomos, com cariótipo bàsicamente semelhante ao das outras espécies de serpentes com 36 cromossomos; *Xenodon merremii*, cujo cariótipo nas células diplóides apresenta só 30 cromossomos e *Clelia occipitolutea*, na qual o número cariotípico é 50 cromossomos, que é o maior número descrito até o presente, nas serpentes.

Com exceção de *Boa constrictor amarali* e *Epicrates cenchria crassus*, os cromossomos sexuais podem ser fàcilmente identificados nas outras espécies, devido ao seu heteromorfismo nas células das fêmeas, que constituem o sexo heterogamético.

As metáfases somáticas, utilizadas para o estudo comparativo da massa cromossômica e do cromossomo X em diferentes espécies, foram selecionadas de preparações citológicas obtidas em condições semelhantes. Tôdas as células provém de culturas temporárias de leucócitos, sendo as preparações citológicas feitas de acôrdo com as técnicas já descritas anteriormente e que compreendem tratamento hipotônico das células durante dez minutos. A coloração foi a de orceína acética ou Giemsa. A seleção das metáfases utilizadas obedeceu aos seguintes critérios: cada cromossomo deveria estar uniformemente corado, bem delineado e com as cromátides irmãs claramente separadas. A célula deveria estar bem achatada e os cromossomos no mesmo plano focal. Por êsses critérios pudemos eliminar as variações decorrentes principalmente dos diferentes graus de hidratação sofridas pelas células durante a preparação citológica.

As medidas foram feitas da seguinte maneira: microfotografias de cinco metáfases foram selecionadas para cada espécie. Os negativos foram feitos com os aumentos 400 × e 2.200 ×. Os filmes utilizados foram o Microfile e o Contrast Process Film, ambos da Kodak. O negativo era colocado no ampliador e a imagem projetada numa fôlha de papel branco ("Victoria", Ind. Brasileira ou "Plover Bond", U.S.A.). O aumento final era de 6.300 ×. Os limites dos cromossomos eram desenhados e as imagens recortadas e pesadas numa balança analítica.

Os recortes dos cromossomos Z e W eram pesados separadamente. A percentagem do Z no lote haplóide (Z : AZ) foi calculada individualmente para cada uma das cinco células e a média aritmética foi determinada. O pêso do lote haplóide dos autossomos foi calculado da seguinte forma:

$$A = \frac{(\text{pêso total}) - (\text{pêso do } Z_1 + Z_2 \text{ (ou W)}}{2}$$

Pesando-se recortes de papel de 63 × 63 mm. que correspondem à área de 100 μ^2 (10 μ × 10 μ) obtivemos um valor médio de 0,3458 g (0,3332-0,3640) para o papel "Victoria" e 0,3332 g (0,3220-0,3440) para o "Plover Bond". Pelo uso dêsses fatôres de conversão, as áreas cromossômicas do Z e do lote diplóide puderam ser calculadas pelos pêsos dos recortes.

As médias dos valores encontrados para a área cromossômica total, assim como, para os cromossomos sexuais Z e W e as suas proporções em relação ao lote haplóide, estão sumarizadas na Tabela que segue.

Na coluna correspondente ao pêso total dos cromossomos, os números entre parêntesis representam os valores mínimo e máximo encontrados em cada espécie. A média dos pêsos totais das sete espécies é de 0.2744 g. A segunda coluna foi obtida por conversão da média do pêso total em área, em cada espécie. Comparando-se as áreas totais das sete espécies investigadas, verificamos que em média êsse valor é de 81.04 μ^2, sendo que o limite de variação entre os valores extremos é de aproximadamente 7 μ^2 (78,81-85.86 μ^2).

Na coluna correspondente ao cromossomo sexual Z, o pêso médio dêsse cromossomo nas sete espécies é de 0,0128 g que corresponde em área a 3.78 μ^2. Nas duas espécies de boídeos incluídas consideramos, para efeito de pesagem, o quarto par de cromossomos, por analogia às espécies de colubrídeos e crotalídeos com 36 cromossomos, onde o cromossomo Z corresponde ao quarto par em ordem de tamanho. A relação entre o cromossomo Z e o lote haplóide (Z : AZ) é de 9,38% em média, nas várias espécies estudadas.

Na coluna correspondente ao cromossomo sexual W, a média dos pêsos encontrados foi de 0,0127 g, que corresponde em área a 3.78 μ^2, à semelhança do valor encontrado para o cromossomo Z. A relação entre o cromossomo W e o lote haplóide é de 9,41%, em média, variando porém de 6,46% na *Xenodon merremii* a 18,20% na *Clelia occipitolutea*.

Nas espécies de CROTALIDAE estudadas, *Bothrops jararaca* e *Bothrops atrox*, as áreas do cromossomo W foram de 2,85 μ^2 e 2,5 μ^2, respectivamente, enquanto que as áreas correspondentes do cromossomo Z foram de 9.57 μ^2 e 8,30 μ^2. Êsses resultados estão de acôrdo com as observações de Schreiber, Menin, Cavenaghi e Fallieri (57). Êsses autores estudaram, por método citofotométrico, as diferenças sexuais no conteúdo em ADN, dos núcleos de eritrócitos de ofídios. Num grupo homogêneo de seis espécies do gênero *Bothrops*, verificaram que o teor de ADN é sempre maior nos machos do que nas fêmeas. Algumas espécies apresentaram esta diferença estatìsticamente significante (*Bothrops jararaca*, *Bothrops cotiara* e *Bothrops jararacussu*); nas demais (*Bothrops atrox*, *Bothrops alternatus* e *Bothrops neuwiedi*) a diferença não foi significativa, mas sua tendência foi no sentido esperado, isto é, de que o macho, sendo o sexo homogamético (ZZ), apresenta maior quantidade de ADN que a fêmea, que é o sexo heterogamético (ZW).

TABELA — MEDIDAS NOS OFÍDIOS EM μ^2, DA ÁREA CROMOSSÔMICA DIPLÓIDE TOTAL, DOS CROMOSSOMOS SEXUAIS Z E W E PROPORÇÕES Z:AUTOSSOMOS E W:AUTOSSOMOS

Espécies (2n)	Área total		Cromossomo sexual Z			Cromossomo sexual W		
	Pêso (g)	μ^2	Pêso (g)	μ^2	Z:AZ (%)	Pêso (g)	μ^2	W:AW (%)
Bothrops jararaca * (CROTALIDAE 2n = 36)	0,2689 (0,2542) (0,2941)	80,70	0,0128 (0,0110) (0,0155)	3,84	9,57	0,0095	2,85	6,59
Bothrops atrox ** (CROTALIDAE 2n = 36)	0,2778 (0,2574) (0,3111)	80,34	0,0117 (0,0096) (0,0150)	3,38	8,30	0,0087	2,5	6,86
Spilotes pullatus anomalepis ** (COLUBRIDAE 2n = 36)	0,2758 (0,2440) (0,3077)	79,76	0,0109 (0,0090) (0,0138)	3,15	7,84	0,117 (0,104) (0,130)	3,38	8,74
Xenodon merremii * (COLUBRIDAE 2n = 30)	0,2626 (0,2537) (0,2813)	78,81	0,0156 (0,0137) (0,0182)	4,68	11,46	0,0083 (0,0068) (0,0100)	2,49	6,46
Clelia occipitolutea * (COLUBRIDAE 2n = 50)	0,2654 (0,2476) (0,2892)	79,65	0,0131 (0,0125) (0,0136)	3,93	10,37	0,0252 (0,0226) (0,0270)	7,56	18,20
			Cromossomo n.º 4					
Boa constrictor amarali * (BOIDAE 2n = 36)	0,2737 (0,2400) (0,3092)	82,14	0,0130 (0,0113) (0,0140)	3,90	9,61			
Epicrates cenchria crassus ** (BOIDAE 2n = 36)	(0,2969) (0,2496) (0,3363)	85,86	(0,0125) (0,0105) (0,0143)	3,61	8,49			

* 10 μ × 10 μ (100 μ^2) = 0,332 g ** 10 μ × 10 μ (100 μ^2) = 0,3458 g.

Determinamos também, para efeito de comparação, a área total cromossômica em duas outras espécies pertencentes a subordens diferentes de répteis: *Anolis carolinensis* (**LACERTILIA**), cujo número diplóide é de 36 cromossomos e *Amyda ferox* (**CHELONIA**), cujo número diplóide é de 66 cromossomos. Encontramos para a primeira espécie uma área total de 82,41 μ^2 e para a segunda de 79,92 μ^2. Êsses valores, apesar da variação do número diplóide, são semelhantes entre si e por sua vez também semelhantes aos determinados para os ofídios.

A área total cromossômica em tôdas as espécies estudadas é pràticamente constante, independente da variação do número de cromossomos do cariótipo. Isso sugere que os rearranjos estruturais, envolvendo pouca ou nenhuma perda na quantidade total de material genético, parecem ter desempenhado um papel importante na especiação dêsses animais. Espécies próximas com diferentes números de cromossomos exemplificam bem êsse fenômeno (58). Mesmo rearranjos mais complicados raramente parecem representar uma perda ou ganho substancial de material genético. Assim por exemplo, em três espécies de macacos do Velho Mundo, o número cromossômico diplóide varia de 42 a 60, mas os valores médios de ADN são essencialmente os mesmos (59).

Na célula diplóide de serpentes, a área total de cromossomos (81 μ^2) corresponde aproximadamente à metade do valor determinado para os mamíferos placentários (155 μ^2). No entanto, o valor absoluto da área do cromossomo Z dos ofídios é sòmente 15% menor que o determinado para o cromossomo X dos mamíferos.

As observações nos mamíferos e nos ofídios sugerem que o cromossomo X ou Z, que aparentemente acumula fatôres governando o desenvolvimento do sexo homogamético, tende a reter, dentro de cada grupo, o tamanho original, através da evolução.

Microcromossomos

Newcomer e Brant (43) estudando a espermatogênese de *Gallus domesticus*, em material fixado por uma mistura a base de ácido propiônico, concluiram que o número de cromossomos nessa espécie não é constante e é muito menos elevado do que o descrito, em 17 raças de galinha, por Yamashina (26), que encontrou 77 cromossomos na fêmea e 78 no macho. O êrro nas avaliações numéricas seria devido ao comportamento aberrante dos microcromossomos, que se formariam durante a prófase a partir de segmentos heterocromáticos dos macrocromossomos. Os microcromossomos seriam supernumerários, teriam um centrômero difuso e se multiplicariam por simples fragmentação. Ao fim duma divisão êles seriam novamente reabsorvidos pelos grandes elementos. Segundo os autores, na pré-prófase do espermatócito primário, massas heterocromáticas seriam visíveis no núcleo e progressivamente condensar-se-iam através do zigóteno e paquíteno, agrupando-se ou localizando-se entre os cromossomos, obscurecendo freqüentemente as relações cromossômicas. Êsses corpúsculos heteropicnóticos positivos poderiam ainda ser observados em associações com os cromossomos através do paquíteno e diplóteno, mas gradualmente condensar-se-iam coalescendo freqüentemente no início da diacinese num número variável de estruturas cromossomóides, às vêzes, com formações aparentes de quiasmas. Segundo Newcomer (60), também na prófase mitótica os microcromossomos não existiriam como entidades individuais, agregando-se para formar massas heteropicnóticas positivas.

Em contraste com essa descrição, Ohno (27), confirmando investigações anteriores de van Brink (17) distinguiu perfeitamente os microcromossomos como tais em tôdas as prófases mitóticas de gônadas embrionárias de *Gallus domesticus* e segundo êsse investigador êles nunca se apresentam com heteropicnose positiva. Na fase de paquíteno, os elementos menores do grupo de microcromossomos podem ser reconhecidos como bivalentes individuais e também nessas células não apresentam heteropicnose positiva. Os microcromossomos retêm sua individualidade e não são heteropicnòticamente positivos em qualquer estágio da mitose, ou da meiose.

Nossas observações em diferentes espécies de cobras levam-nos a concluir que nos ofídios, à semelhança do que van Brink (17) e Ohno (27) demonstraram na galinha, os microcromossomos são realmente estruturas cromossômicas com comportamento idêntico ao dos macrocromossomos e só diferindo dos mesmos pelo tamanho. Mantém sua individualidade durante tôdas as fases da divisão celular e não apresentam heteropicnose positiva quer na mitose quer na meiose. Nas células de ofídios, em que os cromossomos não se apresentam muito condensados, distinguimos perfeitamente a existência de uma constricção correspondente ao centrômero nos microcromossomos. Portanto, não é aceitável a hipótese do centrômero difuso imaginada por Newcomer e Brant, que não identificaram a presença de um centrômero individualizado nos microcromossomos da galinha.

Na mitose final de certas metáfases, parte ou todos os microcromossomos apresentam as duas cromátides já separadas. Êsse fenômeno é, porém, perfeitamente explicável, levando-se em consideração que devido ao seu tamanho reduzido as cromátides dos microcromossomos separam-se antes que as dos macrocromossomos. Além do mais, é freqüente encontrar-se nas metáfases também macrocromossomos com as duas cromátides completamente separadas. Êsse aspecto não serve, portanto, para diferenciar os microcromossomos dos cromossomos verdadeiros como pretendem Newcomer e Brant.

Verificamos que a utilização de técnicas citológicas não apropriadas para o estudo dos cromossomos de ofídios poderiam conduzir a resultados confusos. Empregando técnicas de cortes histológicos em parafina ,os microcromossomos, devido ao seu tamanho reduzido, não são às vêzes incluídos no corte, o que pode dificultar a determinação precisa do seu número. O mesmo pode acontecer com a técnica de esmagamento que, por rompimento mecânico das células, pode resultar na perda de um ou mais cromossomos com distorsão na determinação do número correto de cromossomos existentes no cariótipo. Por outro lado, verificamos que a técnica de coloração empregada também influi nesse aspecto. Certos corantes que, às vêzes, coram com pouca intensidade, como por exemplo o de Feulgen, ou que descoram após algum tempo, como o azul de toluidina, podem dificultar a observação dos microcromossomos, que pelo seu porte reduzido apresentam freqüentemente resolução crítica ao microscópio óptico comum. Corantes como a fucsina básica, quando não apropriadamente utilizados, resultam numa fusão aparente de cromossomos muito próximos e no caso dos microcromossomos podem aparentar coalescência. Como a constituição cromossômica das aves é comparável à dos ofídios, no que diz respeito à existência de microcromossomos, é possível que as técnicas usadas por Newcomer e Brant tenham levado êsses autores a conclusões incorretas quanto à natureza dos microcromossomos.

Uma hipótese que explicaria a origem dos microcromossomos é de que êstes derivariam de fragmentos provenientes da fusão cêntrica, de cromossomos acrocêntricos. Através de um mecanismo "Robertsoniano" a fusão de dois acrocêntricos daria origem a um metacêntrico. Poderíamos imaginar que em decor-

rência dessa translocação surgisse um pequeno fragmento portador de centrômero que viesse a constituir um microcromossomo. Essa hipótese, no entanto, não é satisfatória para explicar os nossos achados em espécies nas quais ocorrem sòmente macrocromossomos acrocêntricos mas que possuem ainda assim considerável número de microcromossomos. *Corallus caninus* (BOIDAE), cujo cariótipo tem 44 cromossomos na célula diplóide, apresenta 24 macrocromossomos todos acrocêntricos e 20 microcromossomos. Em *Clelia occipitolutea* (COLUBRIDAE), cujo cariótipo tem 50 cromossomos, êsse aspecto é ainda mais acentuado, pois, nessa serpente a célula diplóide apresenta 14 macrocromossomos todos acrocêntricos, com exceção do cromossomo sexual Z, e 36 microcromossomos. Portanto, nessas espécies, apesar de não ocorrerem macrocromossomos metacêntricos, o número de microcromossomos não é menor que o encontrado nas espécies em que ocorrem vários metacêntricos, como por exemplo. *Boa constrictor amarali* (BOIDAE), *Bothrops jararaca* (CROTALIDAE) e *Spilotes pullatus anomalepis* (COLUBRIDAE).

Evolução do Cariótipo

Antes de considerar o mecanismo e evolução cariotípica nos ofídios, é interessante examinar sob um ponto de vista geral a relação do número de cromossomos com outros fatôres de significação taxonômica ou evolutiva. Segundo Swanson (61), os dados existentes não permitem no reino vegetal ou animal uma correlação direta entre o número de cromossomos e a posição filogenética. Essas correlações, quando existentes, são geralmente válidas sòmente nos limites da família ou do gênero.

Quando se trata de mudança do número cromossômico e o fator poliploidia pode ser excluído, o problema principal é o de determinar o mecanismo que leva à essa mudança. Uma maneira teórica de explicar êsse fenômeno foi sugerida por Darlington (62). O esquema proposto é de que o ganho e perda de cromossomos de um lote básico envolve ganho ou perda de centrômeros e o êxito dessa mudança dependeria da existência na região adjacente ao centrômero de heterocromatina ou eucromatina.

Mudanças do número básico em *Drosophila* foram extensivamente investigadas e indicam que reduções no número são freqüentes enquanto aumentos são raros, sòmente sendo conhecida em *Drosophila trispina* (n = 7). O número básico primitivo seria 6, compreendendo 5 bastonetes com centrômero subterminal e um cromossomo em forma de grânulo. Essa configuração é encontrada em espécies como *Drosophila virilis*, *D. funebris*, *D. repleta* e *D. tripunctata*. A grande maioria de espécies, porém, mostra um complemento modificado e as variações aparentemente teriam decorrido de translocações (incluindo as fusões cêntricas), de inversões pericêntricas e de alterações da quantidade de heterocromatina (63).

A importância do processo de fusão cêntrica foi evidenciada já em 1916 (64) por Robertson e é porisso também denominada por alguns autores como "lei ou regra de Robertson". Êsse autor mostrou que nos ortópteros os cromossomos metacêntricos poderiam ser considerados como resultado de fusão apical de dois cromossomos acrocêntricos não homólogos. Êsse conceito, que foi verificado num grande número de casos, previa, no entanto, a existência de cromossomos com centrômero terminal (telocêntricos). As fusões cêntricas podem, atualmente, ser consideradas como um tipo especial de translocação, envolvendo os braços inteiros de cromossomos acrocêntricos e parecem ser peculiares do reino animal.

Em *Drosophila*, as fusões cêntricas são possíveis e mais prováveis que outros tipos de translocações talvez por causa dos grandes blocos de heterocromatina na região do centrômero. A perda de um centrômero e da sua heterocromatina adjacente não deve aparentemente produzir efeito marcante na viabilidade quando comparado com a perda de eucromatina. Nos casos de animais híbridos interespecíficos, em que é impossível uma análise meiótica ou de cromossomos do tipo glândula salivar, não se pode verificar se os cromossomos metacêntricos provieram de fusão ou de inversão pericêntrica, a menos que os comprimentos dos braços nesses elementos possam ser usados para distinguir entre os dois tipos de aberração.

Estudos recentes de Wahrman nos mantídeos do gênero *Ameles* constituem um bom exemplo de uma relação "Robertsoniana", que se manifesta tanto intra como interespecìficamente. As populações de três espécies de mantídeos do gênero *Ameles* são heterogêneas em relação aos complementos cromossômicos dos seus membros. Em cada espécie êsse polimorfismo depende de um ou vários cromossomos, que podem estar representados como um metacêntrico ou dois acrocêntricos. Os números cromossômicos diplóides variam de 18 a 29, mas o número total de 30 braços permanece constante assim como a quantidade média de ADN por núcleo (65, 66). Também nos mamíferos, casos bem documentados de filogenia cromossômica confirmando a hipótese de Robertson têm sido relatados, por exemplo, no roedor do gênero *Gerbillus*(58). A translocação do tipo fusão cêntrica parece prevalecer como fator de mudança cromossômica nos animais, enquanto que nas plantas a maioria das translocações analisadas envolvem sòmente uma parte e não todo o braço.

Mudanças podem também ocorrer se um cromossomo em forma de *V* se quebra para formar dois cromossomos em bastonete. Não é sabido, porém, nesse caso, se o centrômero extra deriva de um acessório ou se o centrômero original fragmentou-se para dar dois centrômeros menores, mas ainda funcionais. Centrômeros terminais são instáveis, pelo menos em milho, mas é possível que a estabilidade possa ser adquirida, em certos organismos (67).

Outro tipo de mudança, que introduz variações cariotípicas, é o ganho ou perda de heterocromatina. Onde consideráveis quantidades de heterocromatina são encontradas no cariótipo, alterações na forma, tamanho e número de cromossomos podem ocorrer aparentemente sem sacrifício das porções eucromáticas. Como já foi dito, as fusões cêntricas em animais, envolvendo, provàvelmente, só regiões heterocromáticas assim como perda ou ganho de centrômeros com heterocromatina adjacente, não têm efeito drástico no indivíduo. Nêsse sentido, uma certa quantidade de heterocromatina é evolutivamente desejável como fator de segurança, permitindo um grau de variabilidade cariotípica maior que o sistema composto sòmente de eucromatina (61).

Parece, porém, que os rearranjos estruturais dos cromossomos com pequena ou nenhuma mudança na quantidade total de material têm função muito importante na evolução dos animais. Nos organismos capazes de reprodução sexual, a formação de mediocêntricos a partir de acrocêntricos resulta em diferença no número de grupos de permutação, nos índices de recombinação e nas posições relativas de certos genes. Rearranjos estruturais dêsse tipo podem determinar diferenças nos valores adaptativos de seus portadores.

Segundo White (68) as mudanças estruturais, que são positivamente selecionadas, seguem o que pode ser denominado de "princípio da translocação homóloga", isto é, cromossomos passam pelo mesmo tipo de modificação na mesma seqüência. Isso torna-se compreensível quando consideramos os cromossomos não

como simples conjuntos lineares de genes, mas, como entidades organizadas com uma seqüência que determina as suas relações com o fuso, as suas permutações e seus efeitos de posição. É, portanto, natural que o rearranjo estrutural, que se estabelece na evolução, deva ser de um ou dois tipos principais em todos os cromossomos de um determinado grupo, resultando assim numa situação muito comum, onde todos os cromossomos ou membros cromossômicos são aproximadamente do mesmo tamanho e têm uma distribuição similar de segmentos heterocromáticos.

Os resultados dos nossos estudos, em vinte espécies das famílias BOIDAE, CROTALIDAE e COLUBRIDAE, quando justapostos aos estudos sistemáticos mais recentes (69), permitem-nos sugerir um esquema de evolução para as espécies por nós investigadas.

Dentre as serpentes que estudamos, a família mais primitiva seria a dos BOIDAE. Nessa família, *Corallus caninus* é a única espécie em que encontramos 44 cromossomos, apresentando as outras 36 cromossomos. Os macrocromossomos de *Corallus caninus* são todos acrocêntricos e poderíamos, à base de um mecanismo "Robertsoniano", imaginar que êsse tipo de constituição cromossômica teria dado origem a cariótipos encontrados em outros BOIDAE. Fusões cêntricas de alguns cromossomos, acompanhadas de outras alterações como inversões e translocações, explicariam os cariótipos com 36 cromossomos encontrados nos gêneros *Boa*, *Eunectes* e *Epicrates*. Nenhuma cobra dessa família apresentou heteromorfismo cromossômico em qualquer dos sexos, o que sugeriria um primitivismo do mecanismo cromossômico de determinação do sexo dessas serpentes em relação às que possuem mecanismo mais diferenciado.

Nos COLUBRIDAE, as espécies estudadas dos gêneros *Spilotes*, *Philodryas*, *Dryadophis*, *Drymarchon* e *Chironius*, apresentam 36 cromossomos, semelhantes aos dos BOIDAE. No entanto, nesses gêneros já encontramos heteromorfismo dos cromossomos sexuais na fêmea. O aspecto dêsses cromossomos sexuais parece-nos particularmente interessante. O Z e W, apesar de morfològicamente diferentes, têm tamanho semelhante. Admitindo que uma inversão pericêntrica teria ocorrido num dos homólogos do quarto par, a troca de genes seria reduzida entre êsses cromossomos. Em conseqüência dêsse mecanismo de isolamento os cromossomos poderiam tornar-se cada vez mais diferenciados quanto aos fatôres sexuais. Uma hipótese é de que êste tipo de constituição cromossômica seria dentre os COLUBRIDAE um elo com os BOIDAE Se admitíssemos que as opistoglifas são mais evoluídas que as áglifas, as ser-ao mecanismo cromossômico de determinação do sexo.

Os gêneros *Spilotes*, *Dryadophis*, *Drymarchon* e *Chironius* são constituídos de espécies áglifas, enquanto que o gênero *Philodryas*, cujo cariótipo e cromossomos sexuais são semelhantes, é opistóglifa. Se admitíssemos que as opistóglifas são mais evoluídas que as áglifas, as serpentes do gênero *Philodryas* seriam mais evoluídas que as outras. No entanto, atualmente, muitos sistematas consideram que êsse critério nem sempre é verdadeiro (70).

O colubrídeo *Xenodon merremii* apresenta como número diplóide sòmente 30 cromossomos. Recorrendo ainda ao mecanismo "Robertsoniano" poderíamos imaginar que o seu cariótipo se tenha originado, principalmente, por fusões cêntricas do tipo da constituição cromossômica apresentada pelas espécies de COLUBRIDAE, com 36 cromossomos. Também o grau de heteromorfismo dos seus cromossomos sexuais é maior, pois já teria ocorrido no cromossomo sexual W uma redução de tamanho por eliminação de material cromatínico.

Por sua vez, o colubrídeo *Clelia occipitolutea*, com 50 cromossomos, é a espécie com o maior número diplóide que encontramos. Todos os cromossomos são acrocêntricos, com exceção do cromossomo sexual Z que é metacêntrico. Nessa espécie, excepcionalmente, o cromossomo sexual W é maior que o Z, constituindo o maior cromossomo do cariótipo. Essas características de número diplóide alto e cromossomo W grande, torna difícil a sua correlação com as outras espécies. Talvez o tipo de constituição cromossômica apresentada por *Clelia occipitolutea* decorra de uma linha paralela de evolução cujos cariótipos ancestrais desconhecemos.

Na família CROTALIDAE, que é aparentemente a mais evoluída, as espécies do gênero *Bothrops* e *Crotalus* apresentam 36 cromossomos e, provàvelmente, derivaram dos COLUBRIDAE com número diplóide igual. Essas espécies apresentam acentuado heteromorfismo dos cromossomos sexuais, na fêmea. O cromossomo W resultaria de um W do tipo encontrado, por exemplo, na *Spilotes*, por eliminação de material cromossômico (vide proporções na tabela).

Resumo e Conclusões

O cromossomo Z, nas serpentes apresenta morfologia muito semelhante: a quantidade de material dêsse cromossomo foi avaliada através de um método indireto. O seu valor absoluto é semelhante em sete espécies investigadas e também a proporção do Z em relação ao lote haplóide (Z : AZ) é constante. Pelo mesmo método foi avaliada a quantidade de material de todo o lote cromossômico, encontrando-se valôres similares para as diferentes espécies. Na célula diplóide de serpentes, a área total dos cromossomos corresponde, aproximadamente, à metade do valor determinado para os mamíferos placentários. A constância da área total cromossômica sugere que os rearranjos estruturais acompanhados de pouca ou nenhuma perda na quantidade total de material genético parecem ter desempenhado papel importante na especiação dêsses animais.

Nas espécies de COLUBRIDAE dos gêneros *Spilotes*, *Philodryas*, *Dryadophis*, *Drymarcon* e *Chironius*, os cromossomos sexuais têm o mesmo tamanho, diferindo pelo centrômero, que é submediano no Z e subterminal no W. Êsse aspecto morfológico sugere que tenha ocorrido uma inversão pericêntrica num dos homólogos do quarto par de cromossomos, reduzindo assim consideràvelmente a permutação. Êsse mecanismo de isolamento teria possibilitado um maior acúmulo diferencial de genes relacionados a determinação do sexo nesses heterocromossomos. Nos CROTALIDAE, o tamanho relativamente menor do cromossomo W pode ter decorrido de uma redução por eliminação de material cromatínico.

Em duas espécies investigadas das famílias BOIDAE e CROTALIDAE não foi observado em nenhum dos sexos a existência de um corpúsculo correspondente à cromatina sexual, na periferia interna do núcleo. Também as prófases somáticas e de espermatogônias não demonstraram a existência de qualquer macrocromossomo heteropicnótico. Nenhum dos cromossomos sexuais apresenta heteropicnose positiva, nessas fases. Nos répteis, aparentemente, não existe um mecanismo de compensação de dose para os cromossomos sexuais, como nos mamíferos.

Foi estudada a espermatogênese de *Bothrops jararaca*, observando-se nessa e em outras espécies de ofídios a ocorrência de um número relativamente alto de quiasmas, o que sugere a sua maior semelhança com as aves do que com os

anfíbios que têm número relativamente baixo de quiasmas. A presença nos répteis e nas aves de microcromossomos que não ocorrem, aparentemente, nos anfíbios é também um fator que sugere a proximidade entre os dois primeiros grupos citados.

Nas serpentes os microcromossomos são realmente estruturas cromossômicas com comportamento idêntico ao dos macrocromossomos, dos quais diferem só pelo tamanho. Mantêm sua individualidade durante tôdas as fases da divisão celular e não mostram heteropicnose positiva quer na mitose quer na meiose. Foi identificada nos microcromossomos a presença de uma constricção, correspondente ao centrômero dos macrocromossomos. Em *Bothrops jararaca* verificou-se que o nucléolo encontra-se associado a quatro ou cinco microcromossomos.

Dentre as serpentes estudadas, a família mais primitiva seria a dos BOIDAE. Nessa família, *Corallus caninus* com 2n = 44, sendo todos macrocromossomos acrocêntricos, seria a espécie mais primitiva. Êsse tipo de constituição cromossômica teria dado origem aos cariótipos encontrados nos gêneros *Boa*, *Eunectes* e *Epicrates*.

Nos COLUBRIDAE várias espécies apresentam 36 cromossomos, semelhantes aos dos BOIDAE, mas já possuem cromossomos sexuais heteromórficos. O tipo de constituição cromossômica de *Spilotes*, *Philodryas*, *Dryadophis*, *Drymarchon* e *Chironius* representaria um elo com os BOIDAE. Êsse tipo de constituição teria dado origem ao de *Xenodon merremii*, que tem 2n = 30 e maior heteromorfismo dos cromossomos sexuais. A espécie *Clelia occipitolutea* tem 2n = 50 e um cromossomo W com aproximadamente o dôbro do tamanho em relação ao Z; a sua correlação com as outras espécies de COLUBRIDAE é difícil, decorrendo talvez de uma linha paralela de evolução.

Na família CROTALIDAE, que é a mais evoluída, os gêneros *Bothrops* e *Crotalus* têm 2n = 36 com heteromorfismo acentuado dos cromossomos sexuais, na fêmea. Êsses gêneros derivaram provàvelmente dos COLUBRIDAE, com igual número de cromossomos.

Summary and Conclusions

The Z-chromosomes in snakes are very similar. The amount of material of this chromosome calculated by an indirect method is the same in seven species investigated; the ratio of the Z to the haploid set of chromosomes is also constant. The total amount of chromosome material in the different species is about the same but the chromosome area of a snake cell is only half of that of a placental mammal. The constancy of the total area suggests that structural rearrangements, involving little or no loss in the total amount of genetic material, seems to have played an important role in the speciation of those animals.

In *Spilotes*, *Philodryas*, *Dryadophis*, *Drymarchon* and *Chironius* of the family COLUBRIDAE, the sex chromosomes are of the same size, differing only by the centromere which is submedian in Z and subterminal in W. This aspect suggests that a pericentric inversion had occurred in one element of the fourth pair of chromosomes, crossing-over between the homologues being considerably reduced. This mechanism of isolation resulted in a differential accumulation of sex determining genes in the heterochromosomes. In the CROTALIDAE, the relative smaller size of the W might have happened through elimination of chromosomal material.

In the analysed cells of two species of BOIDAE and CROTALIDAE, it was not possible to identify in either sex what could be considered a sex chromatin body. The somatic and spermatogonia prophases did not reveal the existence of any heteropycnotic macrochromosome. Apparently, a dosage compensation mechanism such as in mammals does not exist in reptiles.

The spermatogenesis of *Bothrops jararaca* was studied. In this and other snake species, the occurrence of a relative large number of chiasmas was observed, suggesting a greater similarity with birds than with amphibians which have a relative smaller number of chiasmas. The presence of microchromosomes in reptiles and birds, and their absence in amphibians, is another factor that suggests a closer relationship between the two first groups mentioned.

The microchromosomes of snakes are actually chromosomal structures with a pattern similar to the macrochromosomes, from which they differ only by size. Their individuality is maintained during all phases of cell division and they are not heteropycnotic, neither in mitosis nor in meiosis. In *Bothrops jararaca* the nucleolus is associated to four or five microchromosomes.

Among the snakes studied, the most primitive species are the BOIDAE. Within this family, *Corallus caninus* with its 44 chromosomes, all of their macrochromosomes being acrocentrics, would be the most primitive species. This type of chromosomal constitution may be related to the one that gave origin to the karyotypes found in *Boa*, *Eunectes* and *Epicrates*.

In COLUBRIDAE several species present 36 chromosomes, similar to those of the BOIDAE, but they already show heteromorphic sex chromosomes. This type of constitution could have originated the one of *Xenodon merremii* which has 2n = 30, and more accentuated heteromorphism of the sex chromosomes. *Clelia occipitolutea* has 2n = 50 and a W-chromosome, which is about twice the size of the Z; its correlation with the other species of COLUBRIDAE is difficult, perhaps due to a parallel line of evolution.

In the family CROTALIDAE, the most evoluted, the genera *Bothrops* and *Crotalus* have 36 chromosomes with an accentuated heteromorphism of the sex chromosomes in the female. These genera probably originated from the COLUBRIDAE with similar number of chromosomes.

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15. ACCIÓN DEL VENENO DE *HAPALOPUS LIMENSIS*

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Introducción

Siendo de importancia práctica el estudio del veneno de las arañas en nuestro medio se realizó bajo la dirección del Dr. Vellard, el presente trabajo, llevado a cabo en los laboratorios del Instituto Nacional de Salud del Ministerio de Salud Pública y Asistencia Social.

Pocos son los científicos que se han ocupado del estudio de las arañas venenosas en el Perú. Las principales observaciones fueron realizadas por Escomel, E.; Cavassa, N.; Leon, A.; Pardo Figueroa, E.; Pesce, H.; Lumbreras, H.; Izu, W. y Vellard, J., quienes refieren varios accidentes producidos por la picadura de las arañas, con muchos casos fatales.

Breve reseña de las Theraphosides en el Perú

En los aledaños de Lima: San Bartolo, Quebrada de Pachacamac, se ha encontrado una especie descrita por Vellard, como *Hapalopus limensis*.

Escomel refiere la existencia de una especie de Mygala en la región de Arequipa del género vecino *Ischnocolus*, sin idenficar la especie; no se ha podido confirmar la veracidade de este dato, ya que puede tratarse de una espécie afín o de la misma *Hapalopus*. También se han descrito las especies: *Hapalopus pictus* Pocock, colectadas en Caraz (Ancash); *Hemirrhagus peruvianus*, descrita por Chamberlin, procedente de Huadquiña, Quimochaca, Urubamba (Cuzco) y *Hemirrhagus major* del valle del Cuzco, descrita por el mismo autor.

Dentro de la sub-familia Grammostolinae, Chamberlin ha descrito la especie *Eurypelma aymara*, de Aymas (Apurimac).

Dados descriptivos de *Hapalopus limensis*

A continuación me permito proporcionar algunos datos descriptivos dados por el Dr. Vellard de la especie *Hapalopus limensis* (Ischnocolinae, THERAPHOSIDAE, **ORTHOGNATHA**): El ♂ adulto alcanza de 50 a 60 mm de largo total y la ♀ adulta hasta 70 mm.

Coloración: el cefalotórax y las patas de un color castaño oscuro. cubiertos con pelos dorados o amarillo claro; abdomen de largos pelos rojizos. Patela más tibia del primer par de patas, más o menos iguales al cefalotórax en

ambos sexos. Patela más tibia del cuarto par, mucho más corto que el tórax en el macho y poco más largo que el tórax en la hembra. En ambos sexos el cuarto par de patas más largo que el primero. Cefalotórax un poco más largo que ancho, de forma ovalada, con una foseta torácica, procurvada. Grupo ocular formando un rectángulo, con bordes paralelos, lo que diferencia completamente a esta especie del género *Hemirrhagus*. Los ojos anteriores en línea procurva, los medianos separados entre sí por su diámetro y un poco más de los laterales; los medianos anteriores un tercio mayores que los laterales anteriores; los laterales posteriores iguales a los laterales anteriores, de los cuales están separados por menos de su semi-diámetro; ojos mediano-posteriores alargados, en forma de coma. Escópulas de los tarsos de las patas del primer y segundo par enteras; escópulas del tercero y cuarto par divididas completamente hasta su base por una fuerte línea de cerdas, lo que permite incluir esta especie en el grupo de los ISCHNOCOLINAE. La tibia del primer par de patas del macho casi recta, apenas ligeramente encurvada en su parte inferior y un poco más gruesa en el ápice, con dos espolones ventrales, uno interno casi recto y otro externo, arqueado, doble del anterior; el tarso se dobla sobre el dorso externo del espolón exterior. La división de las escópulas de los tarsos posteriores, la forma del grupo ocular, la fórmula ocular, permiten incluir esta especie en el género *Hapalopus*. Se diferencia de *Hapalopus pictus*, por su gran tamaño y por su colorido distinto.

Aparato venenoso

Los bordes del artículo basal estan provistos de 11 dientes pequeños en el adulto.

Las dimensiones medias de los quelíceros son: Largo del diente 8 mm, ancho máximo del diente 2 mm, largo de la basal 12 mm, ancho de la basal 8 mm, largo total de los quelíceros 20 mm.

Las dimensiones medias de los quelíceros son: largo del diente 8 mm, vecinos, son fáciles de descubrir a la disección. Se dividen en dos partes distintas: la glándula propiamente dicha, ligeramente encurvada en forma de media luna, colocada en la convexidad de la parte basal y el canal excretor, que sale del polo anterior de la glándula, atraviesa después los dientes, donde termina cerca a la punta de los quelíceros.

Las glándulas estan rodeadas de poderosos grupos musculares, cuya contracción ayuda a vaciar su contenido en el momento de la mordedura. Las glándulas se encuentran siempre libres, sin adherencias con músculos vecinos. La inoculación del veneno es voluntaria y la araña puede morder sin inyectar su veneno. Largo de la glándula: 6 a 7 mm.

El veneno se presenta bajo el aspecto de gotitas límpidas, más o menos viscosas, ligeramente refringentes, solubles en el agua, muy solubles en las soluciones salinas fisiológicas, dejando por evaporación un polvo amarillento. Las glándulas trituradas con suero fisiológico dan una suspensión opalescente. El pH del veneno es ácido (5.4), el del suero, alcalino (7.8).

Biologia

La postura se realiza en los meses de Enero, Febrero y Marzo, encontrándose durante el día ejemplares juveniles después de esta época. Su alimentación es de pequeños miriápodos, escorpiones, coleópteros, lagartijas y algunas arañas.

Viven debajo de las grandes piedras; nos fué fácil capturarlos en los lugares pedregosos durante los meses de Agosto a Noviembre, mientras caminaban sobre las piedras. Sólo conocemos las áreas en que hemos realizado nuestras colecciones, como son: San Bertolo, Quebrada de Pachacamac y Matucana. Creemos que puede existir en otros puntos de la costa del Perú.

Material y métodos

Los ejemplares, 40 Mygalas, de las cuales 30 machos, 4 hembras y 6 juveniles, fueron capturados en lugares pedregosos cerca de los alrededores de Lima como: San Bartolo, Quebrada de Pachacamac, fueron colocados cada uno en un frasco grande de boca ancha, donde permanecieron en reposo durante 15 a 20 días a fin de que las glándulas se encontrasen llenas de veneno en el momento de extraerlas, porque debido a la captura pierden cierta cantidad de veneno al defenderse.

Fué necesario sacrificarlas con cloroformo o éter; después de muertas, los quelíceros fueron seccionados en su base con tijeras muy finas, con la ayuda de un microscopio estereoscópico. Las glándulas separadas de los quelíceros fueron secadas rápidamente en un papel filtro, para quitarles el suero; fueron abiertas en cápsulas de vidrio completamente estériles, y el veneno diluído en solución fisiológica de ClNa al 7.5 $^0/_{00}$; se inoculó diferentes soluciones de veneno, utilizando las vías siguientes: intramuscular, intradérmica, endovenosa y empleando los siguientes animales: cobayos, conejos, perros, palomas. Después de la inoculación se procedía a la observación del animal; sus reacciones sintomatológicas, si presentaba reacción local. Luego de producida la muerte, se procedía a la autopsia de cada animal, observándose el lugar de la inoculación y los órganos internos afectados; esto se realizaba macroscópicamente y microscópicamente, mediante cortes histológicos.

Se han realizado dos formas de experiencias: por picada directa y por inoculación de soluciones de veneno.

Los estudios histopatológicos de la glándula se han hecho utilizando las siguientes técnicas: fijación en solución de Zenker, inclusión en parafina, cortes, coloración con hematoxilina eosina, tricrómica de Pollak, van Gieson y argéntica de Wilder.

Resultados

Por picada directa:

Un ratón de 20 g es mordido en el cuerpo sin precisar el punto de inoculación, por un ejemplar grande de *Hapalopus limensis*. El período de excitación inicial comienza a los 2'; el animal, con fuertes convulsiones y temblor, se mueve por todos lados; demuestra escozor en el hocico. Este período de excitación es seguido 5' después por paresia de los miembros posteriores, acompañada de temblor anterior y pérdida de actividad; a los 7' la paresia alcanza la parte anterior del cuerpo; 8' la parálisis es casi total y a los 9' muere el animal con débiles convulsiones.

Un cobayo de 300 g es mordido en el hocico por un ejemplar adulto de *Hapalopus limensis*. El período de excitación empieza a los 3', seguido de agitación locomotora, con fuertes gritos y un poco de hiper-excitabilidad, a los 15'

comienza la paresia de los miembros posteriores que se acentúa rápidamente; a los 22' la parálisis es casi completa; el animal apoya el hocico sobre el suelo, con las cuatro patas abiertas; la respiración cada vez se hace más lenta; a los 27', cesa completamente la respiración y el animal muere a los 28' con una parálisis rápida de tipo curare.

Por inoculación de soluciones de veneno:

Cobayos: Se utilizaron 7 cobayos de diferentes pesos, a los cuales inoculamos soluciones de veneno de distintas dosis por vía intramuscular, conforme el cuadro n.º 1. *Resultados:* 1) La inyección de ½ glándula de veneno de arañas adultas o juveniles es siempre mortal para el cobayo y la muerte se ha observado en estos animales entre 35' y 108' como promedio; se han presentado casos de 12 y 19 horas, tratándose de arañas juveniles. 2) Con dosis superior a una glándula, la muerte se produce en pocos minutos. 3) La autopsia mostró congestión pulmonar intensa, con grandes zonas hemorrágicas, congestión intensa de las visceras abdominales; líquido hemorrágico en el peritoneo; suprarrenales muy congestionadas, sangre fluída que se coagula lentamente al contacto de los tejidos.

CUADRO I — Cobayos inyectados por via intramuscular

N.º de serie	Peso (grs.)	Araña (edad)	Glándulas	Solución (ClNa)	Resultado	Tiempo
1.º	510	Adulta	½	0.5 ml	murió	53'
2.º	515	Adulta	1	1.0 ml	murió	35'
3.º	425	Adulta	1	1.0 ml	murió	108'
4.º	525	Adulta	1 ½	1.0 ml	murió	17'
5.º	525	Juvenil	½	1.0 ml	murió	12-19 horas
6.º	330	Juvenil	½	1.0 ml	no murió	——
7.º	535	Juvenil	1	0.5 ml	murió	6-12 horas

Conejos: Se han empleado 6 conejos de distintos pesos, a los cuales inyectamos diferentes dosis de veneno, por las vías intradérmica (en la oreja) y endovenosa (vena marginal). La vía intradérmica se ha utilizado en 4 animales para ver la reacción local (cuadro n.º II). *Resultados*: 1) Por vía intradérmica la mínima mortal es de ½ glándula de veneno para conejos de 2 kilos y 1 glándula de veneno por via endovenosa. 2) Con dosis inferior a la mínima mortal los síntomas son muy poco marcados. 3) La inyección intradérmica no produce reacción local. 4) Autopsia: al abrir la cavidad abdominal, todas las vísceras de color oscuro; los vasos mesentéricos muy dilatados, llenos de sangre fluída; 20' después de la muerte, la sangre se coagula muy lentamente al contacto de los tejidos. Un poco de líquido hemorrágico en el peritoneo, vejiga

QUADRO II — Conejos inyectados

N.º de serie	Peso (grs.)	Araña (edad)	Glándulas	Solución (ClNa)	Via	Resultado	Tiempo
1.º	1300	Adulta	¼	2.0 ml	Intradérmica	no murió	——
2.º	1830	Adulta	¼	2.0 ml	Intradérmica	paresia	60'
3.º	1500	Adulta	½	0.25 ml	Intradérmica	murió	52'
4.º	1800	Adulta	¾	2.0 ml	Intradérmica	murió	50'
5.º	1390	Adulta	½	0.25 ml	Endovenosa	no murió	——
6.º	1800	Adulta	1 ½	1.0 ml	Endovenosa	murió	9'

dilatada llena de orina, hígado rojo oscuro, sangrando mucho al corte, riñón oscuro, suprarrenal normal, edema pulmonar muy ligero, fuerte congestión meníngea.

Perros (cuadro n.º III): *Resultados:* 1) Por vía endovenosa la mínima mortal es de 1½ a 2 glándulas de veneno para perros que pesan entre 5 y 8 kilos. 2) A los 3' vomitos; 6' paresia de los miembros posteriores; 10' paresia general; el animal cae de costado, con grandes dificultades al respirar; 12' consigue levantar la cabeza y mover un poco los miembros anteriores; 15' fuerte contractura diafragmática, con parálisis casi total; 30' parálisis de la respiración, el corazón late débilmente; 33' algún movimiento respiratorio; 36' muerte. Tres horas después de la muerte no hay rigidez cadavérica. Autopsia: congestión intensa de todos los órganos abdominales, con un color vinoso; el hígado y el vaso oscuros; vejiga vacía; congestión pulmonar intensa; sangre fluída en todos los vasos.

CUADRO III — Perros inyectados por via endovenosa

N.º de serie	Peso (grs.)	Araña (edad)	Glándulas	Solución (ClNa)	Resultado	Tiempo
1.º	5600	Adulta	1 ½	1.0 ml	murió	36'
2.º	6000	Adulta	2	1.0 ml	murió	9'
3.º	8500	Adulta	2	1.0 ml	no murió	——
4.º	6200	Adulta	4	1.0 ml	murió	11'

Palomas: Hemos empleado cuatro palomas a las cuales inyectamos soluciones de veneno por vía endovenosa (cuadro n.º IV). *Resultados:* 1) Por vía endovenosa la dosis mínima mortal es de ¾ de glándula de veneno. 2) Con dosis inferior a la mínima mortal los síntomas son bien marcados, pero el animal

se restablece. 3) A los 2' parálisis de las patas, cayéndose al suelo, 5' caída de las alas, la cabeza cae sobre el suelo por parálisis de la nuca, los ojos cerrados; el animal trata de abrir el pico para respirar mejor, muerte a los 12'. Autopsia: congestión intensa de las vísceras abdominales y torácicas; sangre parcialmente coagulada en el corazón.

CUADRO IV — Palomas inyectadas por vía endovenosa

N.º de serie	Peso (grs.)	Araña (edad)	Glándulas	Solución (ClNa)	Resultado	Tiempo
1.º	400	Adulta	1	2.0 ml	murió	12'
2.º	375	Adulta	¾	2.0 ml	murió	30'
3.º	300	Adulta	½	2.0 ml	no murió	——
4.º	350	Juvenil	1	1.0 ml	murió	5'

Con los animales de sangre fría no se hizo mayores experiencias, por haberse utilizado sólo dos especies de animales, un sapo, *Bufo spinulosus limensis*, al que se inyectó 2 glándulas em 2 ml., con resultados negativos y una lagartija *Tropidurus peruvianus*, al que se inyectó ½ glándula en 0.5 ml, síntoma: ligera paresia, restableciéndose a los 15'.

Desde el punto de vista de su acción sobre el sistema nervioso, el veneno de *Hapalopus limensis*, posee los caracteres generales de los venenos neurotóxicos. La sintomatología es uniforme en todos los animales, es una curarización típica, iniciándose por paresia, seguida de parálisis rápida y una muerte por parálisis respiratoria; el corazón después de la parálisis respiratoria continua latiendo uno o varios minutos. Cualquiera que sea la vía de inoculación, la muerte es siempre rápida.

Histología de la glándula

Se advierten los cortes una cubierta muscular de forma espiralada estriada, fácilmente perceptible, de regular grosor, netamente separada, sobre la cual se observa el epitelio constituído por una hilera de células basales de las que desprenden estrías citoplasmáticas, que, a manera de largos filamentos, avanzan hacia la luz de la glándula.

En la base de estas prolongaciones toman asiento núcleos que tienen el mismo aspecto de los epiteliales. La presencia de una membrana basal es neta. En algunas zonas próximas a la membrana basal se advierte una acumulación circunscrita de gránulos muy finos y que parecen corresponder a los de la secreción.

Descripción de las células epiteliales: Se las ha visto constituyendo una simple hilera celular (epitelio monoestratificado), núcleo ovalado con cromatina densa. A la hematoxilina el carácter nuclear cromatínico podría calificar-se de tipo cromatínico "pulverulento".

El protoplasma de bordes imprecisos que nos permite deslindar los límites intercelulares, toma el aspecto general de condensaciones fibrilares que en forma de largos filamentos se proyecta hacia la luz de la glándula.

La capa muscular es oblícua con fascículos de diversas direcciones.

La estructura glandular se acerca al tipo merocrino, donde las traveras persisten aprisionando en sus mallas al contenido de la secreción glandular y la destrucción celular sólo alcanza a las células centrales de la glándula.

Anatomia Patológica

Todos los animales muestran congestión intensa de las vísceras.

Los estudios histopatológicos se han hecho en cobayos, conejos y perros.

COBAYO N.º 5: Hígado — congestión, especialmente marcada en los grandes vasos (estasis). *Piel* — en algunos sitios, junto al punto de inoculación, pérdida de la sustancia córnea y del epitelio germinativo. *Testículos* — con imagen funcional de reposo. Se observan los canalículos seminíferos completamente llenos de células germinales y de espermatozoides. *Corteza cerebral* — ligera congestión vascular. *Pancreas* — este órgano conserva sus caracteres morfológicos normales en lo que respecta a su posición exocrina, así como en su posición insular. *Pulmones* — congestión vascular especialmente marcada en los pequeños vasos; muchas cavidades alveolares repletas de células de aspecto exudativo. *Bazo* — Conserva sus caracteres histológicos propios.

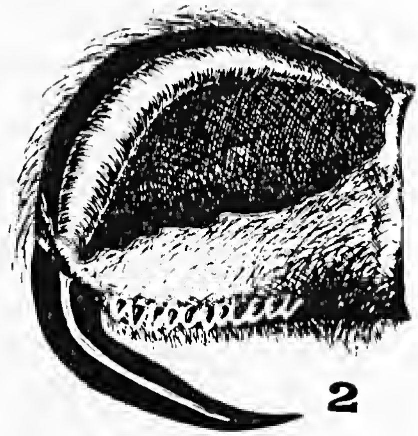
COBAYO N.º 3: Riñon — la congestión vascular es intensa, exibiendo en ciertos segmentos del órgano (zona medular) focos hemorrágicos. *Cerebro* — caracteres normales. *Pulmones* — intensa congestión, marcado edema de las paredes alveolares.

CONEJO N.º 4: Riñon — Se nota en los glomérulos aumento del número de núcleos; histológicamente, se observa edema en las paredes de los tubos. Algunas de las pequeñas áreas de la zona medular se muestran con infiltración celular junto a los tubos del asa de Henle. Igualmente, algunos tubos contorneados vecinos a algunos glomérulos se observan infiltrados. En la zona medular las infiltraciones celulares parecen ser periféricas a veces sanguineas de mediano calibre. *Pulmón* — conservación de los caracteres histológicos normales. *Cerebelo* — normal. *Hígado* — se observa en el hígado pequeños focos de infiltración en las proximidades de los vasos peri-lobulillares.

PERRO N.º 1: Riñon — congestión de algunos capilares glandulares e incremento del número de núcleos en los corpúsculos de Malpighi. En la zona medular de las pirámides se nota moderada congestión vascular. Algunos tubos contorneados proximales se presentan con su epitelio de aspecto hidrópico. *Hígado* — ligera infiltración alrededor de los vasos centro-lobulillares, los mismos que se ofrecen con aspecto congestivo y con discreto edema de sus paredes. La infiltración corresponde a elementos celulares del tipo linfócito. *Capsula suprarrenal* — conserva sus caracteres histológicos normales.

Como resultado de la observación de los órganos pertenecientes a los animales de experimentación, puede decirse que los afectados son el riñón, el hígado y el pulmón con lesiones de tipo inflamatorio agudo, representadas principalmente en forma de transtornos congestivos vasculares, sin cambios degenerativos en los respectivos parénquimas. Órganos como el sistema nervioso central, cerebro, cerebelo y el bazo no exiben cambios ostensibles.

1



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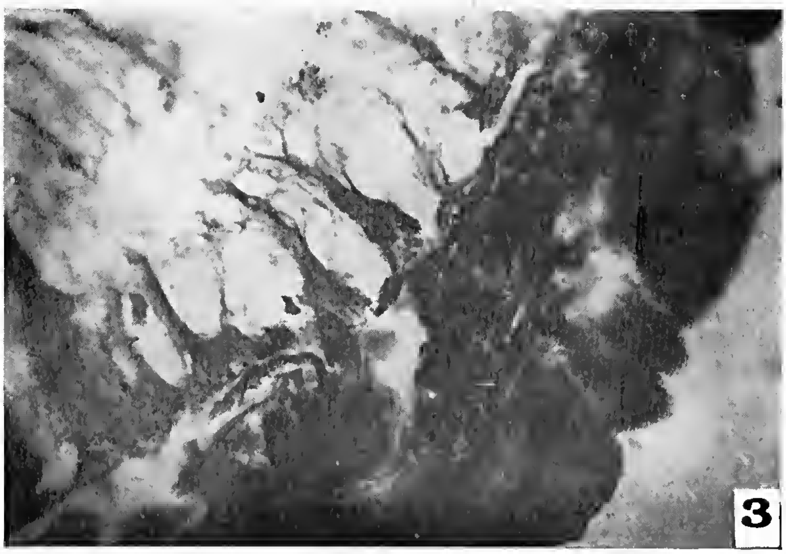

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Fig. 1 — *Hapalopus limensis* — Hembra.

Fig. 2 — *Hapalopus limensis* — Posición de la glándula de veneno dentro del quelícero.

Fig. 3 — *Hapalopus limensis* — Corte de la glándula de veneno (aum. de 500 × aproximadamente).

Summary

Experimental injections with the venom of *Hapalopus limensis* in guinea-pigs, rabbits, dogs, pigeons, toads (*Bufo spinulosus limensis*) and lizards (*Tropidurus peruvianus*) showed the following results:

1. The venom is, principally, of neurotoxic action.
2. The first symptom observed is paresis, followed by death caused by respiratory paralysis, observed in all laboratory animals.
3. In the hot-blooded animals, death occurs in a short-time, generally less than an hour, in any inoculation route.
4. In the cold-blooded animals, when the venom is injected in the ventral lymphatic sack of the toad and intraperitoneally in the small lizard, the action is transitory.
5. The minimum lethal doses are: for 400-500 gr. guinea-pigs, intramuscularly, 0,5 gland; for two kilo rabbits, intradermically, 0,5 gland and endovenously, 1 gland; for 5 to 8 kilo dogs, endovenously, 1,5 to 2 glands; for 375 to 400 gr pigeons, endovenously, 0,75 to 1 gland.
6. Intensive visceral congestions and degenerative lesions in the liver, kidneys and lungs can be observed in the autopsy and histo-pathological studies.
7. The venom produces neither immediate nor late local reaction.

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16. CASOS DE LOXOSCELISMO GRAVE OCURRIDOS EN LA CIUDAD DE BUENOS AIRES Y ALREDEDORES EN LOS ÚLTIMOS AÑOS

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Los accidentes ocasionados por *Loxosceles laeta* (Nicolet) en la ciudad de Buenos Aires y alrededores se observan con mucho más frecuencia en los meses de calor que en el invierno. La mayor parte de los casos que nos llegan en consulta siguen una evolución favorable limitandose, a los sumo, a la aparición de dolor local seguido de aureola congestiva y a veces de algunas ampollas.

El diagnóstico de esta forma de araneísmo domiciliario debe hacerse la mayor parte de las veces, en base al cuadro que presentan los enfermos, puesto que en muy contadas ocasiones intentan o logran capturar a la araña.

Concordando con Schenone (1), consideramos que dado el conocimiento actual que se posee de la sintomatología determinada por la picadura de *Loxosceles laeta*, no es indispensable su captura y subsiguiente identificación para hacer el diagnóstico de loxoscelismo. En efecto, el diagnóstico diferencial de la lesión cutánea ofrece relativamente pocas dificultades puesto que debe hacerse principalmente con relación a otras dos entidades nosológicas, el carbunco y la erisipela gangrenosa. En cuanto al compromiso sistémico, habrá que tener en cuenta otras etiologías determinantes de insuficiencia renal aguda, entre las que seguramente ésta es la única que puede asociarse a la típica lesión cutánea y al antecedente de una picadura por artrópodo.

Las pequeñas dimensiones y la poca agresividad de esta especie han originado dificultades y contribuído a demorar la identificación de la verdadera entidad responsable de tales accidentes. De ahí, que a pesar de tratarse de un tipo de araneísmo vastamente extendido en América y conocido desde muchos años atrás, recién en 1935 Escudero (2) sospecha, por la investigación experimental, que sea atribuible a *Loxosceles laeta* (Nicolet) un caso mortal de araneísmo cutáneo; en 1937 Macchiavello (3) la identifica en forma indubitable como causante del araneísmo necrótico-cutáneo y posteriormente en 1947 el mismo autor (4) la responsabiliza también del araneísmo hemolítico, lo que confirman Mackinnon y Witkind (5) en 1953 para el mismo tipo de accidentes registrados en el Uruguay.

Damos a conocer a continuación cuatro casos en los que nos cupo actuar y que presentaron evolución más seria, dos coresponden a la forma cutáneo necrótica hemolítica y los otros dos a la forma cutáneo necrótica simple.

Caso I

M. S. O., niña de 6 años, argentina. Procedencia: Baradero (Provincia de Buenos Aires).

El 15 de febrero de 1954: es picada a las 17.30 hs, por un insecto (?) en región supraclavicular izquierda, en su domicilio.

2.º día: A la mañana presentaba edema blanco con puntillado rojizo, irregular en la zona supraclavicular izquierda.

3.º día: Aparece orina hemoglobinúrica. Más tarde se le transfunden 150 cm^3 de sangre total.

4.º día: Continúa la hemoglobinuria y se presenta tinte subictérico en piel y mucosas. Ante el cuadro de anemia (3.000.000 de hematíes por mm^3) se le transfunden 250 cm^3 de sangre total. Hay colapso circulatorio superficial. Está formada la placa livedoide, que abarca el hemitórax superior izquierdo, cuello y brazo del mismo lado hasta la región deltoidea (Fig. 1). Se administra Cortone y Benadryl inyectable. Posteriormente se transfunden 300 cm^3 de sangre total. Hay hipertermia de 39°C.

Fig. 1 — Caso I. Se observa el lugar de la picadura y extensión de la mancha equimótica.

5.º día: Enferma en anuria, que se prolongará hasta su muerte; se transfunden 200 cm³ de plasma y, por sondeo vesical, se recogen unos 20 cm³ de orina color pardusco. La placa livedoide se presenta dura, de color violáceo y de límites más precisos. Enferma febril, oscilando entre 37°C y 39°C.

6.º día: Se comienza la diálisis gástrica, y continúa el tratamiento con Cortone y Benadryl. Enfermita lúcida; una deposición abundante.

7.º día: Enferma indiferente y deshidratada, la placa marmórea comienza a involucionar, disminuyendo el edema y la intensidad de la coloración. Una deposición diarreica.

8.º día: Actitud indiferente y quejosa. Una deposición diarreica.

9.º día: Enfermita adinámica e indiferente, dos deposiciones diarreicas. Hematocrito 33%.

10.º día: A las 13 hs, aparecen nistagmus y movimientos convulsivos, no responde a las solicitaciones. Deposición diarreica. Vomita reiteradamente. La mancha livedoide con franca tendencia a desaparecer.

11.º día: Se retira la diálisis gástrica. Enfermita no responde, continúan nistagmus y convulsiones. Frecuencia de pulso 110. Presión arterial: Mx 110, Mn 50. Fallece durante el acto operatorio al intentar efetuársele una exanguineo transfusión.

En la figura n.º 2 se sintetiza en un gráfico la evolución del cuadro humoral, señalándose además la duración de la hemoglobinuria y de la anuria.

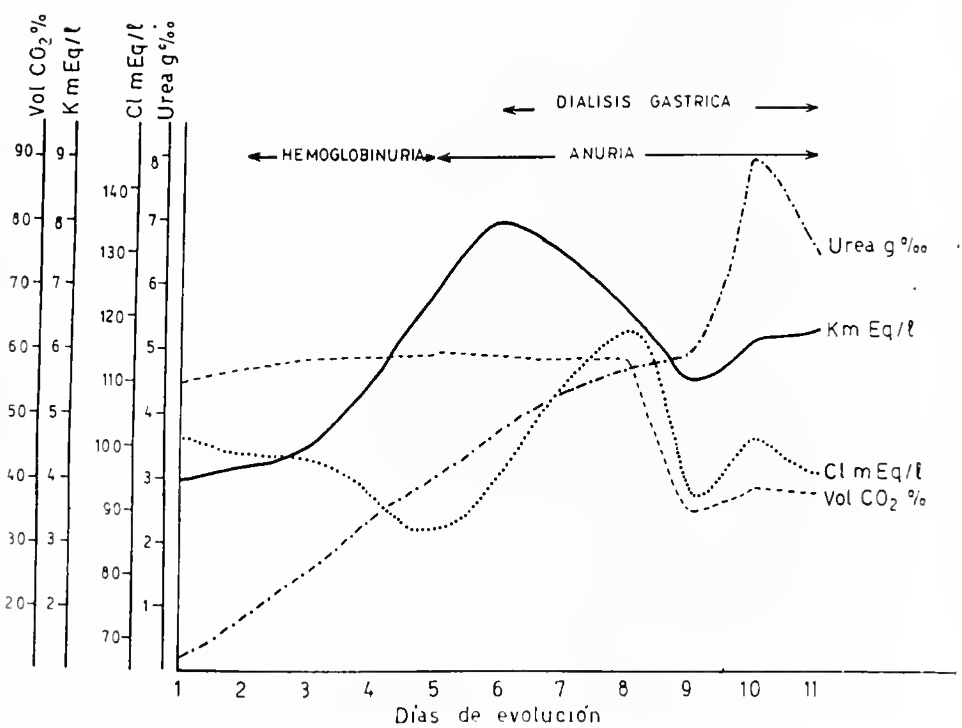

Fig. 2 — Caso I. Evolución del cuadro humoral .

Caso II

J. R. de A., argentina, 38 años, casada, con 3 hijos varones, y embarazo de 3 meses. Procedencia: San Andrés de Giles (provincia de Buenos Aires).

A continuación hacemos un resumen del caso ya publicado (6).

El 24 de enero de 1962, aproximadamente a las 15.00 hs, luego de tomar un baño, al cubrirse con la bata, que había dejado colgada en la pared, siente una picadura en la región subescapular izquierda. Al buscar la causa, encuentra una araña a la que arroja, restándole toda importancia.

Unas 3 horas después comienza un dolor persistente, que va aumentando en intensidad. A las 4 horas del accidente hay además sensación de tumefacción en el hombro y región pectoral.

2.º día: A las 11.00 hs aparece intensa hematuria, que es confundida con una metrorragia, lo que agregado al malestar general, motivó su internación en un hospital de la zona. Se nota coloración amarillenta del tegumento. Hay ligera epistaxis y mucho dolor de cabeza.

3.º día: Sigue desmejorando y queda establecida la anuria. Sospechando el médico tratante la posible etiología del proceso, se pone en comunicación con nosotros. Aconsejamos el inmediato traslado a Buenos Aires. A las 55 horas de la picadura se le inyectan 4 ampollas de suero anti-*Loxosceles* (en total 200 unidades antinecrosantes).

4.º día: Estado actual — Enferma lúcida, ictérica, anémica, disneica y con signos de deshidratación. Tensión arterial Mx 100, Mn 65; con 100 pulsos por minuto. Abdomen distendido y depresible. Espleno y hepatomegalia dolorosa a la palpación. El resto sin particularidades.

La picadura tiene forma numular y diámetro de 2.5 cm con su borde congestivo, y se halla en el centro de la zona de edema equimótico, elástico y doloroso (placa marmórea). Hay infartos ganglionares subaxilar y supraclavicular.

Los datos de laboratorio son los siguientes: Hematocrito: 24%. Hemoglobinemia: 316 mg. Leucocitosis: 20.500 con neutrofilia. Urea: 1.70 g/litro. Bilirrubina directa, indirecta y reacciones de floculación dentro de límites normales. Cl: 102 mEq/l. Bicarbonato: 16 mEq/l. Sodio: 144 mEq/l. Potásio: 5.2 mEq/l.

Evolución: La paciente atravesó una fase oligo-anúrica que duró 22 días, oscilando su diuresis entre 30 y 180 cm^3. Durante esta etapa reflejó mediante una severa anemia el resultado de su importante hemólisis, la que fue corregida cuando sus hematocritos descendían del 25%, con transfusiones de glóbulos desplasmatizados. La potasemia tuvo tendencia a ascender siempre a cifras patológicas, llegando algunas veces a 6.8 mEq/litro a pesar de administrarse diariamente 50 gramos de resinas. El bicarbonato tuvo descensos graduales y paralelos a la elevación de la urea y prolongación de la anuria. La creatinina se mantuvo alrededor de los 15 mg.

Durante estos 22 días de oligo-anuria, la paciente fue sometida a una exanguino-transfusión y dos diálisis (Fig. 4).

No fue posible evidenciar toxinas hemolíticas en las muestras de sangre extraída con ese objeto.

La paciente fue dada de alta a los 35 días, hallándose en la etapa de poliuria compensadora. Durante el tiempo de su internación no hubieron indicios sugestivos de interrupción de embarazo y a su alta un urocitograma no aclaró el cuadro y una reacción de Galli-Mainini dió resultado positivo.

A los cuatro meses y medio de embarazo el útero presentaba la altura correspondiente y la madre percibía movimeintos activos del feto. Los controles efectuados permitieron valorar la buena evolución de la paciente.

Fig. 3 — Caso II. Muestra el lugar de la picadura y extensión de la placa livedoide.

El parto se produce a los 8 meses justos, espontáneo; resultando un feto femenino hipotrófico, de 1.850 g de peso.

La niña ha evolucionado normalmente hasta el presente.

La escara final resultó notablemente pequeña, alcanzando apenas a 1 cm de largo al tiempo de desprenderse.

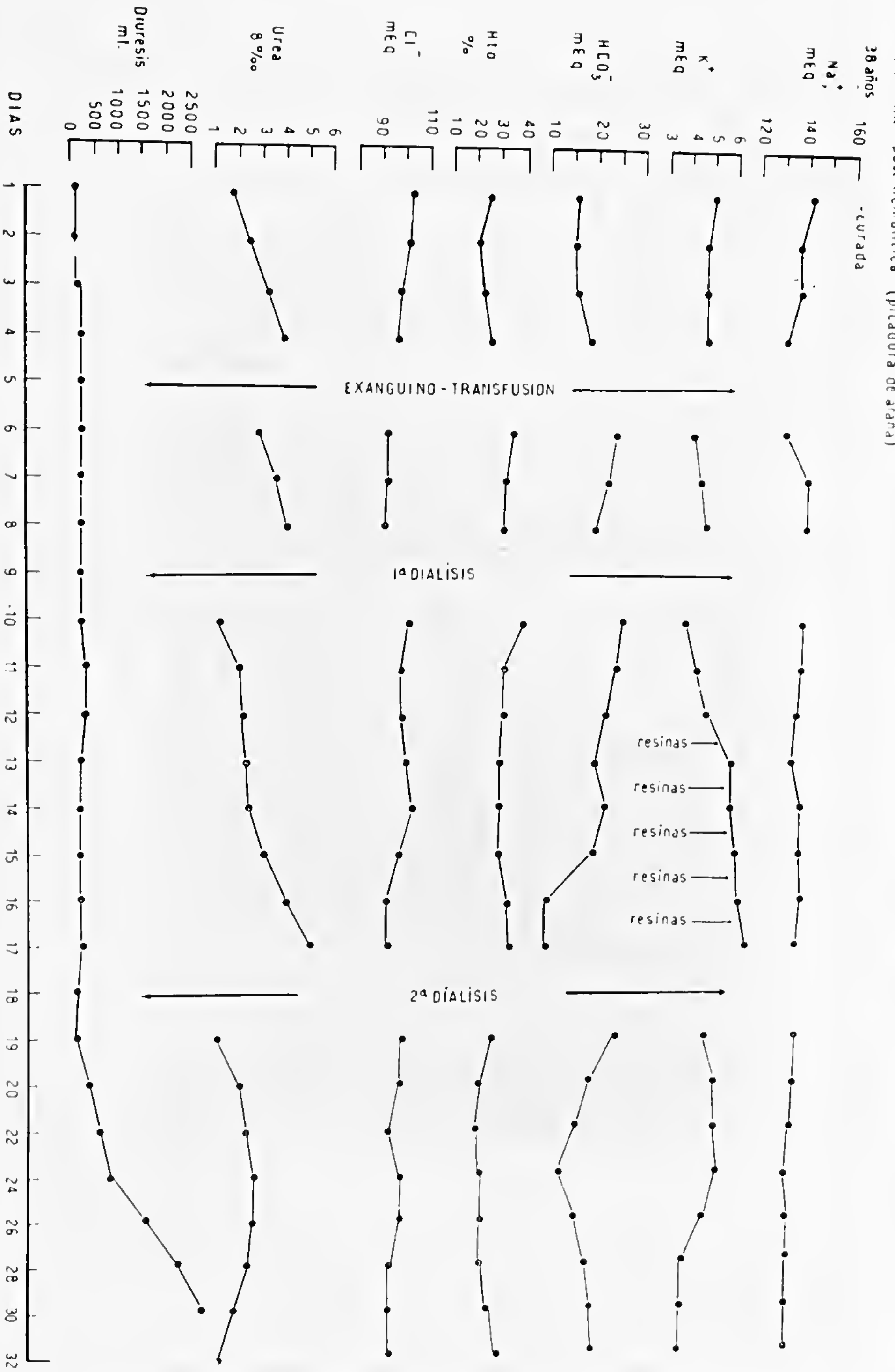

Fig. 4 — Caso II. Evolución del cuadro humoral.

Días después de la picadura fueron traídos 7 ejemplares de arañas capturadas en el cuarto de baño de la casa (que era de construcción reciente), resultando todas corresponder a una única especie: *Loxosceles laeta.*

Caso III

V. N. T., argentina, 2 años. Procedencia: Villa Dominico (Avellaneda). provincia de Buenos Aires.

El 18 de octubre de 1964 a la mañana experimenta dolor y aparece una pequeña manchita por arriba de la rodilla iszquierda.

2.º día: Hipertermia moderada, excitación; la lesión local toma el aspecto de una pustulita.

3.º día: Aparición de una gran mancha marmórea en la parte interna del muslo. Al desprenderse la costra de la ampolla pustulosa fluye un líquido seroso. Se administran antibióticos.

8.º día: La mancha se extiende aún más hasta la ingle, asentándose sobre un edema duro. Continúa tratamiento con antibióticos.

9.º día: Sin mayores modificaciones. Continúa el tratamiento con antibióticos. Es internada en un Servicio de Pediatría con el diagnóstico presuntivo de tromboflebitis de la vena femoral. La mancha equimótica toma la parte interna y se extiende hacia la cara anterior del muslo izquierdo y a través de la ingle sobre el pubis (Fig. 5). Todo ello se asienta sobre un edema duro que

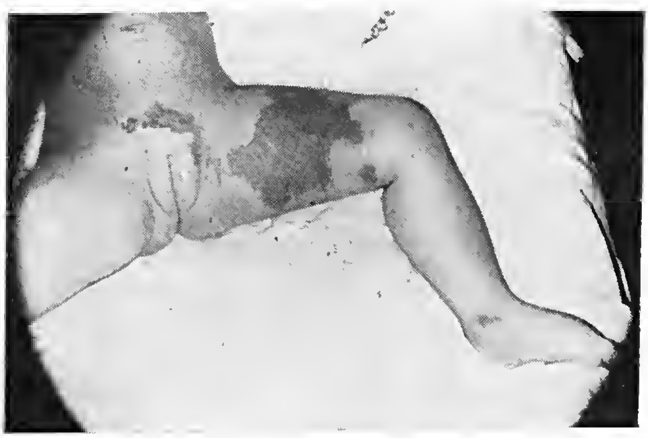

Fig. 5 — Caso III. Dia 10.º. Mancha equimótica.

se extiende desde el bajo vientre hasta el muslo y en menor proporción a la pierna y tobillo del mismo lado. Aparece ligero tinte subictérico y orinas aparentemente no hematúricas. Continúa tratamiento con antibióticos y antihistamínicos. Se diagnostica loxoscelismo y se le administran 2 ampollas (100 unidades antinecrosantes) de suero anti-*L o x o s c e l e s* (Instituto Butantan). Hiperleucocitosis: 37.000. Hematocrito: 35%. Bilirrubinemia: 0.30 g%. Uremia: 0.40 g%. Potasio: 4.5 mEq/l. Sodio: 120 mEq/l.

10.º día: Enfermita quejosa. Lesión local sin mayores modificaciones. Hematocrito: 28%. Bilirrubinemia: 0.90 g%. Uremia: 0.38 g%. Potasio: 5.4 mEq/l. Sodio: 113 mEq/l. Cloro: 92.5 mEq/l.

11.º día: Más animada, comienza a alimentarse espontáneamente, se le sigue administrando suero por fleboclisis, y antibióticos. Hielo local.

12.º día: Buen estado general. Comienza a aparecer una línea oscura que bordea la placa violácea. Hematocrito: 28%. Bilirrubinemia: 0.40 g%. Potasio: 4.1 mEq/l. Sódio: 143 mEq/l. Cloro: 98 mEq/l.

13.º día: Continúa buen estado general. Se acentúa la demarcación señalada.

22.º día: Comienza el desprendimiento de los bordes de la escara del muslo y del pubis. Ha habido hipertermia de 40°C. Se continúa con antibióticos.

23.º día: Buen estado general. Continúa el desprendimiento de los bordes de la escara. Hipertermia de 38°C. Continúa antibióticos.

28.º día: Buen estado general. El desprendimiento de los bordes de la escara incluye piel y celular subcutáneo, presentándose los labios evertidos, mientras la escara principal permanece firme en el centro (Fig. 6).

Fig. 6 — Caso III. Día 28.º. Labios evertidos en la lesión principal.

Posteriormente ingresa a un Servicio de Cirurgia Plástica, adonde previo desprendimiento de los restos de la escara (quedando la aponeurosis expuesta) y toilette de la herida, se le hacen injertos en tira según técnica de Thierch. Es dada de alta a los dos meses.

Durante gran parte de su evolución fue tratada con antibióticos y mientras estuvo internada no se observaron alteraciones en la orina ni disminución notoria de la diuresis.

Caso IV

A. B., argentina, 25 años. Procedência: Capital Federal. Si bien este caso no adquirió la gravedad de los anteriores, lo damos a conocer porque se pudo seguir su evolución prácticamente desde el momento de la picadura.

El 16 de noviembre de 1965 a las 02.00 hs, en una confitería del centro de la ciudad de Buenos Aires, al apoyar el brazo sobre el tapado dejado sobre una silla, siente un fuerte pinchazo en mitad del brazo izquierdo, lado inferior, que le hace levantar el brazo bruseamente, cayendo al suelo una pequeña araña. Cuatro horas después hay fuerte dolor en el brazo, y poco más tarde observa una pequeña ampollita con contenido seroso. El dolor aumenta y a las 17.00 llega hasta el pectoral. Concurre a un servicio hospitalario, donde le administran un antialérgico. Apenas durmió por la noche. No observó fiebre ni orina roja.

2.º día: Es vista por nosotros. Presenta todo el brazo edematizado y rojizo. En el lado interno inferior se destaca una mancha blanquecina, de unos 12 × 6 cm, de bordes irregulares. No hay indicios de ampollas, y en el lugar de la picadura apenas se nota una pequeña mancha más oscura, de unos 2 mm. Dolor quemante en todo el brazo y especialmente donde está la mancha blanquecina. Estado general sin nada de particular. Se aplican dos inyecciones anti-*Loxosceles* (Instituto Butantan; 100 unidades anti-necrosantes).

3.º y 4.º días: Se vuelven a administrar 4 ampollas más, es decir que en total se aplicaron 6 ampollas o 300 unidades.

5.º día: El dolor sigue muy intenso. No hay temperatura, ni albúmina, ni señales de hemoglobinuria.

6.º día: La mancha rojiza se extiende hasta la muñeca por el lado externo y rodea casi el brazo a la altura del codo. Sobre la mancha blanquecina (livedoide) se va formando una zona azulada. Todo el brazo edematizado (Fig. 7). El dolor sigue intenso y continuo. Apirexia y orina normal. Se palpan ganglios axilares dolorosos.

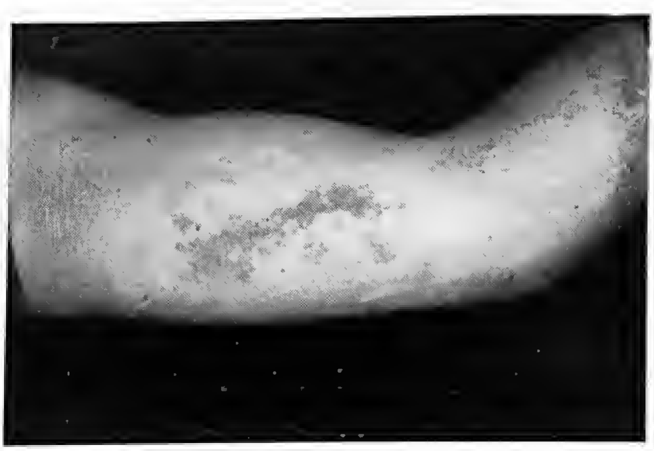

Fig. 7 — Caso IV. Dia 6.º. Formación de la mancha livedoide.

9.º día: Brazo totalmente deshinchado; ha cesado el dolor, salvo en el borde de la zona livedoide; la parte central de ésta es insensible.

33.º día: Comienzan a levantarse los bordes más delgados de la escara de 9.5 cm × 3.5 cm (Fig. 8).

Fig. 8 — Caso IV. Día 33.º. Placa necrótica.

40.º día: A la tarde comienza a separarse la escara ya bastante disminuída de tamaño. Hay dolor (Fig. 9).

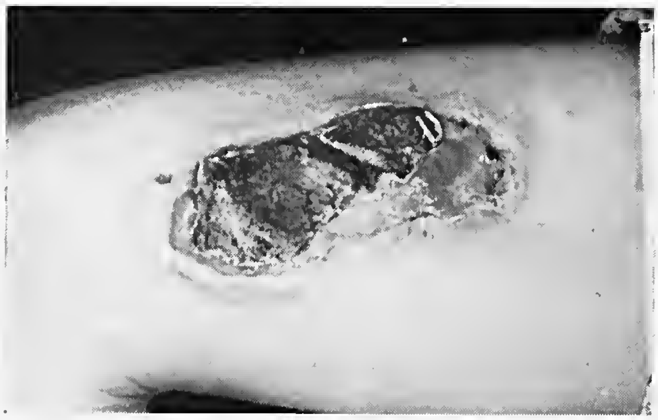

Fig. 9 — Caso IV. Día 40.º. Comienza el desprendimiento de la escara.

55.º día: En un servicio hospitalario le desprenden la escara que apenas estaba unida por el centro, quedando la lesión limpia, granulosa. Es aconsejada la plástica.

Se deja constancia que a pesar del tratamiento precoz y aparentemente suficiente (6 ampollas en total) la evolución fue lenta y terminó en una lesión necrótica.

Summary

Four human cases of loxoscelism, studied from the clinical and therapeutical point of view, are reported. Two were of the simple cutaneous-necrotic type and the other two had a more severe evolution, death ensuing in one of them, and were of the cutaneous-necrotic-haemolytic type.

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17. BEE VENOM: GLANDS, INTOXICATION, ACCIDENTS

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Primitive bees and wasps are solitary animals. Infra social or social behaviour is a relatively recent evolutionary step paralleled by morphological and physiological adaptative changes.

Our purpose in this study is to discuss some of the changes in the sting apparatus as well as to comment on the bee venom and its action.

Bees are **HYMENOPTERA** from the super family APOIDEA. Michener (1) proposed an evolutionary taxonomic tree for this super family (Fig. 1).

The majority of the families systematized are composed of solitary or infra social bees. In the family APIDAE there are three social tribes: BOMBINI, APINI and MELIPONINI. BOMBINI are the "bumblebees"; among the APINI are the honey-bees and the MELIPONINI are stingless bees.

The sting apparatus of BOMBINI and APINI was studied by Dufour (2) and Bordas (3). It is located in the posterior part of the abdomen between the rectum and the ovaries. Rectum, ovaries and the sting apparatus share a common opening. Two glands were described: one spindle shaped, was called "basic", by Dufour. The other, called "acid", is bifid in its distal part, and thread shaped; the two distal segments unite in a common, long, convoluted, thread like tubule, that opens in a pear shaped reservoir, leading through its proximal, slender segment, in the sting chamber (Fig. 2). In *Bombus* (*Fervidobombus*) *atratus* Franklin, the acid gland is more ramified and the common tubule, before opening in the reservoir, is very short.

MELIPONINI cannot sting, but still have a vestigial sting provided with a glandular sac, the size of which, changes from very large, occupying 2/3 of the abdominal cavity in some species, to very small, vestigial structures, in others (4).

The loss of the sting may be interpreted as a useful evolutionary step, since its sheding, as it occurs in the *Apis* genera, after the action of stinging, is followed by death of the insect. The inability to sting preserves the insect and maintains the colony.

Stingless bees are not defenseless bees: many defense mechanisms have been described (5, 6) as the development of powerful mandibles; the ability to hinder flying or walking performances of the enemies through mixtures of wax and vegetable gums that are sticked to their bodies or moving apendices; the emission of bad smelling or bad tasting secretions; massive attack to repel an intruder, and many others.

The appearance of these defense mechanisms lead to the obsolescence of the sting and its eventual atrophy (4).

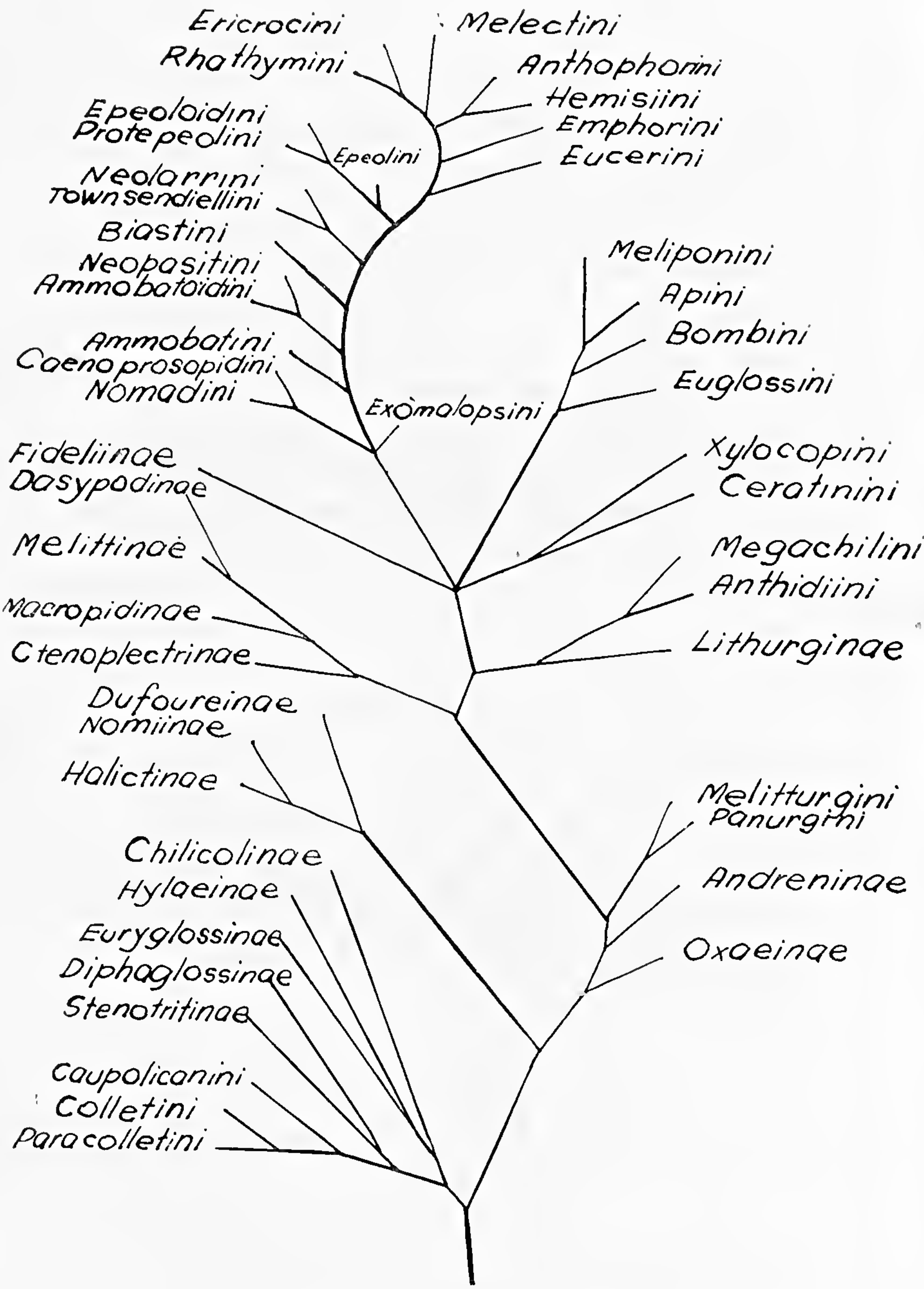

Fig. 1 — Taxonomic tree of APOIDEA according to Michener, 1944.

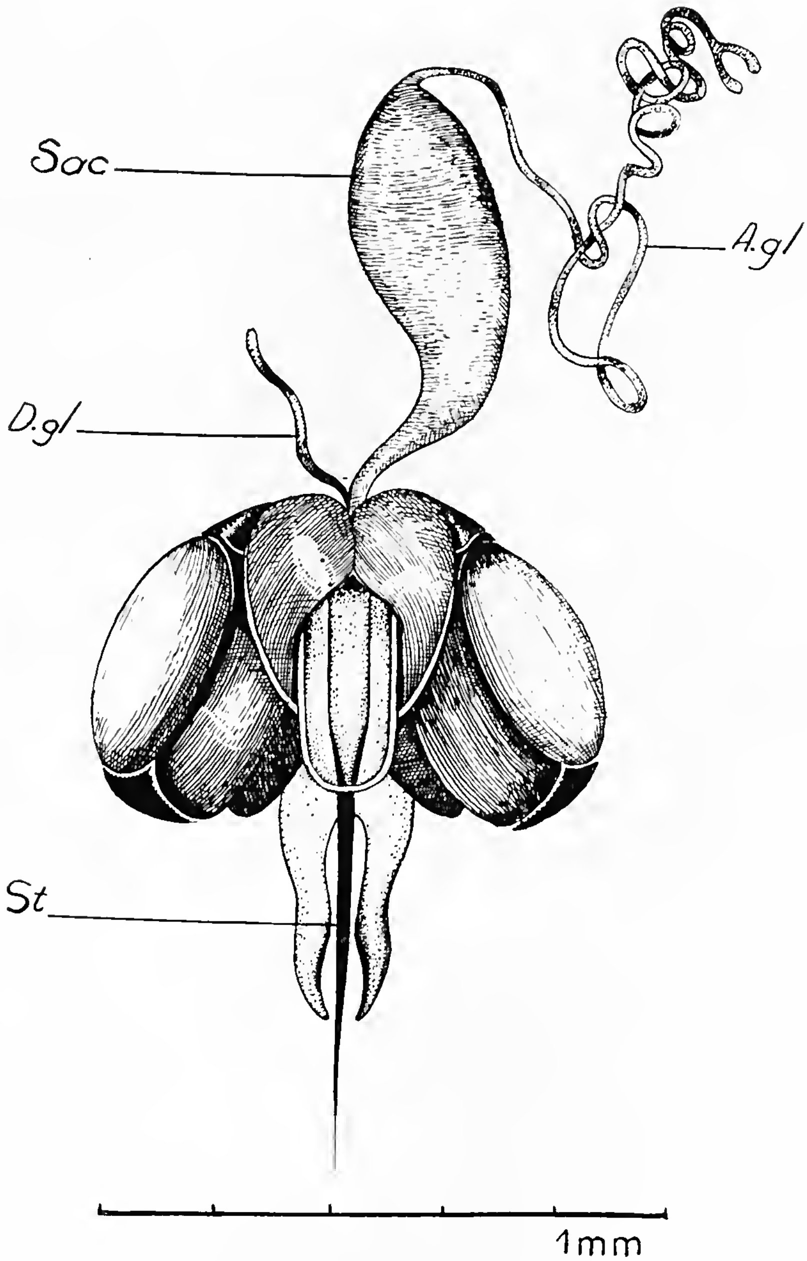

Fig. 2 — Sting accessories of *Apis mellifera adansonii*. A.gl. = Acid gland; Sac = pear shaped reservoir; D.gl. = Dufour gland or "basic" gland; St = sting.

The sting apparatus of Apini

Morphologists, pharmacologists and biochemists have been interested in the sting apparatus of Apini. The first studied its anatomy and histology. The latter, the poison and its consequences.

We began our studies on morphology (4) and could not agree with Dufour (2), Carlet (7) and Bordas (3). They described that, both the acid and basic glands, before opening in the sting chamber, were united in a common duct. Trojan (8), however, described the two glands as opening independently into the chamber. Our observations agree with this last author. In this work he suggests that, in the queen bees, the basic gland secretion, pouring over the eggs, forms a layer around them that serves the double purpose of protecting them and sticking them into the cell walls.

According to Trojan (8) and our own observations, the bee venom is the secretion of the acid gland only and not a mixture of the secretions of the two glands.

Biochemists and pharmacologists studied the poison as eliminated from the sting and not the properties of the secretions of each gland.

The chemistry of the venom

Langer in 1897 (9) considered the bee poison as an essential basic alcaloid. Phisalix in 1922 (10) described three components of the poison; one histiotoxic, one convulsive and the last paralising, each with different physicochemical characteristics.

Reinert (11) identified a protein, rich in triptophane, that could be dialysed into two fractions, one with hemolytic activity, the other neurotoxic. He also said that the inflammatory exudate of the sting contained 1 to 1,5% of histamine. Ackermann and Mauer (12) were of the opinion that histamine was not found in the poison itself but was part of the host reactions.

Feldberg and Kellaway had shown previously (13) the histamine releasing properties of the bee poison.

More recently a dehydrogenase inhibitor and one hyaluronidase have been isolated from the venom (reviewed by Hodgson, 1955) (14).

In 1954, Neumann and Habermann (15) isolated and characterized a basic protein, mellitin, of marked cytolitic activity over mast cells and red blood cells.

Phospholipase A, isolated by Högberg and Uvnäs (16, 17) acts also degranulating rat mast cells.

In 1965, Rothschild (18) studied the release of histamine in rats by phospholipase A and mellitin from the bee venom.

The symptoms and consequences of bee sting

Symptoms and signs of bee sting are very well known by the lay men all over the world. In temperate and cold climates, bee stinging is much more frequent than most of the other poisonous bites or stings by snakes, spiders or scorpions. Despite this, the medical literature on the subject is scarse.

Symptoms may vary from case to case; they usually are more severe when the patient receives numerous stings; they also tend to be more severe after many expositions of the same patient to the poison.

The first symptom is acute, local pain, described as "burning", followed after a few minutes by local swelling and itching; the area shows a small, clear, round, central zone, surrounded by a red halo.

In the case of *Apis mellifera* (honeybee) the sting itself is found in the clear, central zone; it pierces the epidermis and may penetrate 2 to 3 mm in it. Wasps and the majority of the solitary bees, however, do not lose the sting.

The local reaction subsides in a few hours and all the symptoms and signs have usually disappeared after 24 hours.

In a few instances, the consequences are more severe and Thompson (19) ranked them in the following order:

1. Abnormal swelling and irritation, lasting an abnormal time.

2. Massive urticaria and oedema supervening.

3. Shock and collapse or even loss of consciousness with hypotonia and tachycardia, followed by mild pyrexia and sometimes diarrhoea and polyuria.

4. True dyspnoea soon after sting.

5. Sudden severe general symptoms:

   a) hot flush over the whole body;
   b) severe dyspnoea;
   c) wheezing and coughing, almost choking;
   d) general trembling;
   e) great anxiety, sometimes followed by coma. In women this may be accompanied by uterine contractions. This severe condition may last up to three or four days.

6. Death.

Death due to bee sting

The available data about death following bee stinging are not relyable. Swinny (20) reported on seven deaths, in the state of Texas, in 1949; he suggested that this is a conservative estimation, the actual, correct figure being close to 20 to 30 per year in the area studied. Parrish (21) reviewing the mortality due to insect bites or stings in 1950-1954 in the U.S.A. found 84 cases (review of death certificates); from these, 50% were caused by *Apis mellifera ligustica*; at the same time only 71 deaths were ascribed to snake bites.

In Denmarck between 1951 and 1959 bees were responsable for 2/3 of all the deaths ascribed to poisonous animals (22).

All the cited authors were of the opinion that their figures are conservative. Doctors, in general, are very cautious to ascribe death to such a simple and common accident as a bee sting: they rather look for some other more usual cause, as acute cardiovascular disease. Indeed, many authors think that one can better estimate the frequency of deaths after bee sting by reviewing the newspapers than by reviewing death certificates.

Jensen (22) studied 34 cases of deaths due to bee stinging. Fig. 3 and 4 summarize, graphically, his findings. Fig. 3 shows his 22 cases of males and 12 cases of females, by age. Death is rare up to 30 years and the majority of the cases died between 30 and 50 years.

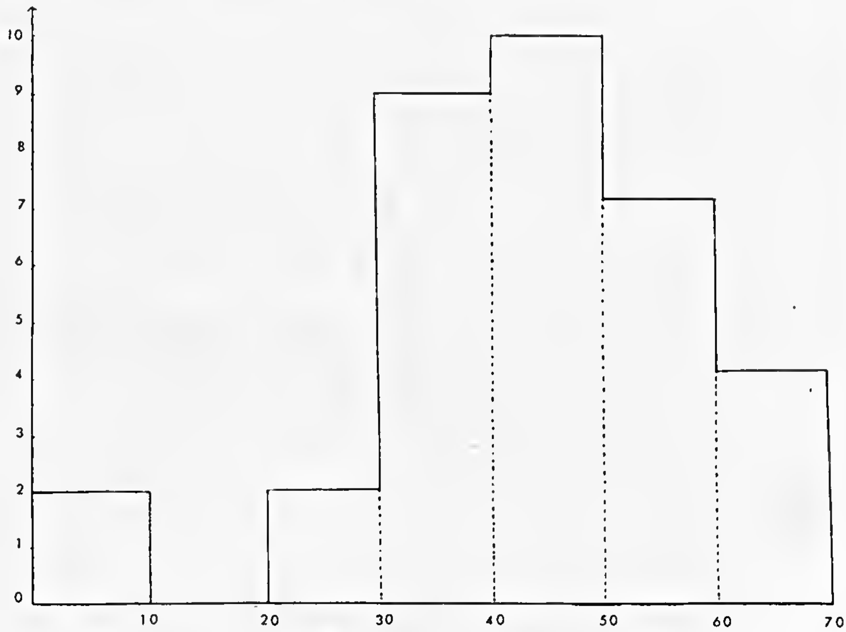

Fig. 3 — Death caused by bee and wasp sting in the U.S.A. according to the age. Data from Jensen, 1961.

Delpech (23) came to the conclusion, that death by bee sting is much more common in adults. From the 34 cases of Parrish (21) only 4 (11,7%) were under 30 years of age; all but five of the cases in his series were perfectly healthy before the accident. The age distribution seems peculiar since children are usually more exposed to bees.

Fig. 4 shows a correlation between death by bee stinging and previous allergic complaints in Jensen cases. In 16 of his 34 cases there was past history of allergic disorders, five had been previously stinged and one had never been stinged before.

The hypothesis that anaphylaxis might be the pathogenetic mechanism of death is atractive. Indeed, death supervenes within one hour of the accident in the great majority of the reported cases. The symptoms are also compatible with the hypothesis being of the same type observed on other types of human anaphylactic shock.

The age distribution of the cases also agrees with the suggested hypothesis. As said above, children are much more exposed to bees than adults; being smaller they should be more susceptible to the poison if one thinks in term of dosage by kg of weight.

Death by other poisonous animals as snakes, spiders or scorpions is more common below 20 years of age (40% between 0 and 19 years). Only 7% of the deaths ascribed to bee sting occur below 20 years of age. In the case of

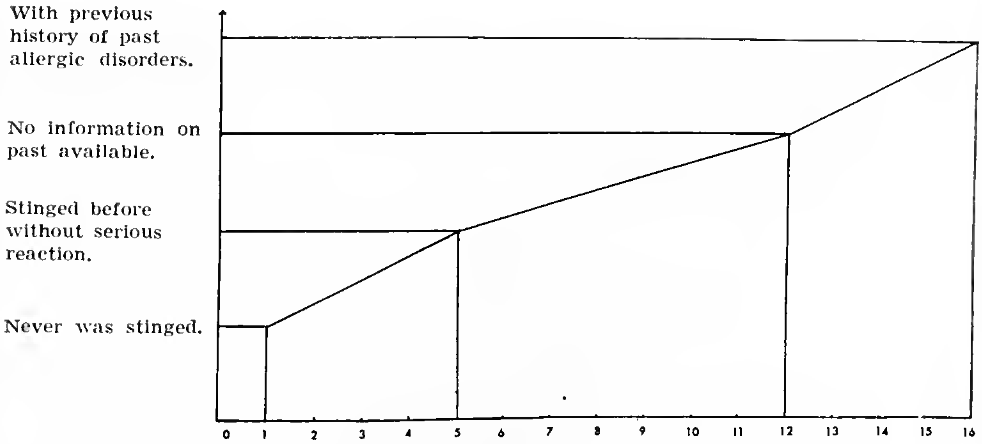

Fig. 4 — Past history of allergy and mortality by bee sting. Data from Jensen, 1961.

snakes, scorpions and spiders there is a definite correlation between dosage per kg of body weight and the severity of the symptoms. We know of 4 beekeepers who have been exposed, in the same day, to 600 to 1500 bee stings who survived, nicely, after the usual local signs and symptoms.

Death by bee sting in Brazil

Up to 1956 our beehives were populated by *Apis mellifera ligustica*, the Italian honeybee. At that time Dr. W. E. Kerr, a geneticist from the "Escola Superior de Agricultura Luiz de Queiroz" in São Paulo. made a trip to Afriea sponsored by the State Department of Agriculture, the University of São Paulo and the Brazilian Association of Beekeepers. His objective was to study the possibility of amelioration of the honey yield of our beehives through imbreeding of our Italian honeybees with the more productive *Apis mellifera adansonii*, an African bee. The "African" bee produces about 400% more honey then the "Italian" bee.

Since last year, newspapers in São Paulo have called the attention to an apparent increase in death due to bee stings and the proposed explanation was the agressivity of the "African" bees. At this moment we are collecting data on the subject and our impression is that the facts are not as serious as to advocate the destruetion of all African colonies, an action that has been proposed and discussed.

It is true that the Afriean bees are more agressive but the data that we have up to now gathered, register cases of death among chicken and swines. Actual danger to men seems to be exagerated in the press.

In the State of São Paulo, with 16 million inhabitants there were 6 certain human deaths between 1964 and 1965. Two of those cases were men over 70 years of age and one was a small child that was abandoned by his sister at the door of a beehive.

In the same period of one year, in the State of Texas, U.S.A., with a population, at that time. of 8 million inhabitants there were 7 definite deaths

caused by bee stings. The bees in Texas are only of Italian or Caucasian origin: no African bees had been imported.

There appears to be no difference in the nature of the poison produced by *Apis mellifera ligustica* and *Apis mellifera adansonii.*

Once the industry of honey develops in any country there is an increase in the number of mortal accidents and this is entirely independent of the cultivated species.

In conclusion, we do not believe that the introduction of African bees in our country is a serious danger.

There is no clear indication that it has increased the risk of a serious damage of men or animals beyond the expected increase of risk in consequence of the rapid development of beekeeping as a profitable industry.

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18. LEPIDOPTERISMO Y ERUCISMO. EPIDEMIOLOGÍA Y ASPECTOS CLÍNICOS EN EL PERÚ

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Aceptamos la convención de distinguir los accidentes tóxicos, locales o generales, causados en humanos por lepidópteros *adultos* con el nombre de LEPIDOPTERISMO y los causados por *orugas* (en latín "eruca"), con el nombre de ERUCISMO.

LEPIDOPTERISMO

Casi todas las observaciones conciernen a Sud América. Casos aislados han sido observados en la Guayana Francesa, en Uruguay, en Argentina; pequeños brotes mayores en el Brasil (Amapá) y en el Perú.

Epidemiología

Los agentes causantes más comunes son lepidópteros de la familia HEMILEUCIDAE, género *Hylesia*. En el Perú la especie causante más común es *H. volvex* Dyar, mariposa de dimensión mediana, cm 5 a 6, color pizarroso, que vive sobre varias plantas y las abandona raras veces.

Las armas vulnerantes son espinas barbadas o "flechitas" de 150 micra, implantadas sobre el abdomen de las hembras, impregnadas de veneno hidrosoluble.

La única región del Perú en donde se ha registrado accidentes masivos es la amazónica. En Tingomaría, pequeña ciudad en la hoya del alto Huallaga, durante años, H. F. Allard observó invasión de mariposas adultas después de terminada la estación de las grandes lluvias, de Abril a Junio: en 1952 registró la invasión mayor, que duró 7 semanas, con apogéo en Mayo. Los vuelos eran vespertinos y nocturnos; millares de mariposas envolvían los focos eléctricos exteriores y frecuentemente penetraban en las casas. El pulvísculo de setas desprendidas era muy abundante. Por lo menos un 70% de los habitantes, o sea más de 3000 personas sufrieron los efectos del envenenamiento; muchos acudieron al Hospital.

En 1966 A. Tejada confirmó estos datos, registró 514 casos en 15 localidades de la hoya del Huallaga, y además estableció lo siguiente: a) en el año existen 2 invasiones (abril-mayo; diciembre-enero) y a veces una tercera (agosto-septiembre); b) se han producido exacerbaciones periódicas por invasiones masivas cada 4 ó 5 años (1947, 1952, 1957, 1962, 1966).

El mecanismo habitual de producción de los accidentes humanos colectivos ha sido "indirecto" por desprendimiento de las setas ponzoñosas que flotaban en la atmósfera, depositándose luego sobre ventanas, muebles y objetos, y de allí a la piel.

Aspectos clínicos

Sintomatología

a) Los accidentes espontáneos se manifiestan como una dermitis que ocupa el cuello, la cara de flexión del codo, y otras zonas de piel delicada. Allí aparece una zona eritematosa, pruriginosa, varias pápulas, y algunas vesículas, que suelen declinar en 6-7 días. La repetición de los accidentes en cada noche agrava estos síntomas y puede mantenerlos por varias semanas.

b) Experiencias efectuadas (1952) en la región sobre pobladores que antes no habían padecido de estos accidentes, mediante la frotación cutánea con el cuerpo de estas mariposas, determinaron en todos a los 15-20 minutos la aparición local de un eritema pruriginoso.

e) Experiencia análoga (1952) con estas mariposas remitidas un mes después al extranjero determinó eritema a las 2 horas; a las 4 horas calor local, pápulas edematosas, prurito; a los 2 días una vesícula y varias petequias. La dermitis aguda declinó a los 7 días y las petequias desaparecieron a los 13 días.

d) A. Tejada (1966) experimentó en 16 personas, en la región y fuera de ella, con resultado análogo.

e) El manejo de mariposas muertas determinó (1966) accidentes extensos en dos entomólogos.

El cuadro clínico observado concuerda con una etiología tóxica por productos del grupo histamínico. Por otra parte la tendencia crónica que acompaña la repetición diaria de los accidentes sugiere algún grado de sensibilización.

El diagnóstico se efectúa por el dato epidemiológico y por la dermitis típica. El no haber tenido en cuenta el primer dato ha dado lugar a falsos diagnósticos de brote de rubeola.

Tratamiento

El uso en la primera media hora de una loción de hiposulfito de sodio al 50% parece neutralizar el veneno "in situ" pues desaparecen las lesiones y el prurito; dentro de 1ª hora hay alguna eficacia; después ninguna.

El uso precoz de antihistamínicos sintéticos por vía oral atenúa rápidamente la erupción cutánea y suprime el prurito; su uso tardío actúa bien sobre el prurito y el consiguiente insomnio.

El uso de corticosteroides ha sido favorable en algunos casos.

Prevención

Durante las invasiones, mantener apagadas las luces desde el ocaso hasta la hora del sueño.

ERUCISMO

Las orugas fanerotóxicas peruanas que hemos observado pertenecen en su mayor parte a la familia MEGALOPYGIDAE.

Tenemos registrados 1383 casos de erucismo individual, directo.

EPIDEMIOLOGÍA

Las Orugas Ponzoñosas del Perú

Las especies predominantes y su frecuencia son:

a) Una *Megalopyge sp.* ("cuy dorado") 28.9%
b) Una *Megalopyge sp.* ("utcu balluca") 16.8%
c) Una *Podalia sp.* ("sarohomé cremoso") 10.2%
d) Una *Megalopyge sp.* ("cuy de fuego") 6.2%

Especies de menor difusión pertenecen a SATURNIDAE, a HEMILEUCIDAE y a SPHINGIDAE. (La descripción morfológica de las orugas, así como el estudio macro y microscópico de su aparato venenoso los hemo efectuado en otro trabajo ya entregado para publicacíon.)

Sinecología

La correlación entre el habitat y el modo de vida de las orugas y los hábitos regionales del hombre tiene dos aspectos:

a) *Ecología de las Orugas*

En el Perú, como en casi toda la región Neotropical, la ecología de las orugas es opuesta a la de las regiones Paleártica y Neártica. El ciclo de vida de los lepidópteros es con frecuencia semestral y aún trimestral. Su habitat preferente es la selva densa con escasos vientos y población sumamente rala. La pérdida de setas, absorbidas por la selva, no reviste importancia. Por lo tanto no hay accidentes colectivos: todos son individuales y *directos.*

La frecuencia cronológica de las orugas puede deducirse del número de accidentes que provocan. La primera serie de 635 casos (1958-64) estudiados en 34 distritos (de 9 provincias) se distribuyen en los meses del año con una proporción promedia mensual del 7%, salvo en el bimestre marzo-abril sucesivo a la "grán" estación lluviosa en que asciende al 12,5% mensual, y en el bimestre septiembre-octubre sucesivo a las "pequeñas lluvias". con 9,6% mensual, totalizando ambos períodos 44,2%.

b) *Ecologia humana*

El "hinterland" de los escasos pobladores es un retazo arrancado a la selva virgen en el que las plantas cultivadas producen todo el año. Los accidentes observados en la citada serie de 635 casos son *individuales* y se produjeron: cultivando y cosechando 59,7%, talando 12,8%, cargando 9,9%. Las plantas infestadas han sido: alimenticias 42,0% y comerciales (cafeto, banana, algodón, tabaco) 35,0%. El accidente directo, en estas zonas, es obligado producto del trabajo agrícola individual. Es casi inevitable: 42,5% de los casos sufrieron de 2 a 5 accidentes y 15,8% de 6 a 15.

Etiopatogenia

El criterio ecológico aplicado a la etiopatogenia nos permite distinguir varios mecanismos:

a) *Erucismo verdadero directo* — Es por contacto directo de una oruga peluda, viva con el tegumento humano de un individuo. En el Perú es la regla.

b) *Erucismo verdadero indirecto* — Las setas de las orugas peludas desprendidas, en el follaje o en el tronco o en el suelo son las responsables de accidentes. En el Perú es la excepción: Burstein y col. (1957) observaron casos alrededor de un árbol "malo".

c) *Para-erucismo* — Es producido por orugas desnudas criptotóxicas, sin aparato vulnerante. No lo hemos observado.

d) *Meta-erucismo* — Las setas larvarias tóxicas son transmitidas a la cocona o hasta al insecto adulto. Lo llamamos erucismo "por encargo". En el Perú se han dado algunos casos.

Aspectos clínicos

Nuestras observaciones de erucismo individual directo se refieren a 2 series: la 1.ª (Pesce y Delgado, 1958-64) con 635 casos; la 2.ª (Pesce y Tejada, 1966) con 748 casos; totalizan 1383 casos.

La primera serie, ya tabulada, con 635 casos comprende 1939 accidentes que se distribuyen así: a) 265 accidentes en 265 personas con accidente único; b) 1674 accidentes en 370 personas con accidentes repetidos. En la tabulación sintomatológica solamente hemos considerado los 635 accidentes cada uno de los cuales ha motivado una historia clínica.

Cuadro clínico

La dermitis local aguda es el componente obligado de todos los accidentes. Si bien en un 40% de los casos ella es el único sindrome y evoluciona en pocas horas (2 a 12), en un 60% de los casos se agrega extensión regional del processo con neuritis difusa (24-36 h), y en un 20-25% de los casos se presentan también signos generales, que en niños y mujeres pueden revestir gravedad.

La sintomatología, sumamente rica y variada, merece por lo menos el siguiente análisis somero:

Sintomatología local y regional

a) Debemos destacar el síndrome dérmico de *tipo histamínico* o histaminoide con eritema (97,1%), edema (65,0%), pápula-habón (36,4%), petequias (27,9%), observando que el prurito aparenta una cifra baja (14,5%) por ser enmascarado por el dolor urente difuso (56,8%).

b) Entre los signos *inflamatorios* de origen tóxico merecen relevarse el dolor ganglionar (32,9%) y el infarto ganglionar (29,0%).

c) El compromiso *neural* es alto: dolor *urente* (56,8%) casi siempre difuso, a veces acompañado por neuralgias; dolor articular (2,7%).

d) Efectos *necróticos* superficiales: flictenas (14,2%).

e) Una *secuela* muy frecuente es la hipereronia residual (64,0%); a veces huellas cicatriciales (2.0%), residuo de flictenas.

Sintomatología general

Su notable frecuencia observada en la selva del Perú contrasta con lo referido por otros autores.

a) Entre los signos generales, hay malestar (21,6%), escalofrío (9,9%) y sensación del alza térmica (31,9%) aunque en parte falseada por el dolor urente difuso, a veces casi generalizado.

b) Hay signos tóxicos compatibles con el tipo histamínico: nauseas (7,1%), vómitos (2,7%), diarreas (2,0%), erupción urticariana (1,4%).

c) Signos circulatorios atribuibles a la misma causa son: cefalea por probable edema cerebral (17,5%), angustia (5,4%).

d) Signos nerviosos son: confusión (2,7%), dromofilia (1,3%).

e) Colapso en sus distintos grados: adinamia (19,1%) postración (3,1%), lipotimia (1.6%).

Aspectos inmunológicos

Consideramos para este efecto los 1939 accidentes registrados en la 1.ª serie de los cuales 1674 son repetidos.

Entre los 370 individuos que han sufrido 1674 accidentes comprobamos que en 82,5% de los casos el último accidente guarda similitud con los anteriores; en 10,4% hubo atenuación marcada; en 7,1% el último accidente fue más serio que los anteriores. Sin embargo 1/3 de estos accidentados atribuyen la mayor seriedad del accidente a la diferente especie de la oruga causante y 1/5 la atribuye a la mayor intensidad del contacto.

Al parecer el veneno de las orugas peruanas tendría tendencia a producir cierto grado de inmunidad. No conocemos casos de sensibilización; aparentemente son muy raros.

Tratamiento

En el medio rural más remoto nuestra experiencia nos indica que la aplicación de compresas húmedas calientes y la inmersión segmentaria en agua caliente produce rápida remisión de la dermitis, de la neuritis y de la sintomatología general, en lapsos entre 1 y 3 horas según la seriedad del caso.

Donde ha sido posible, la ingestión de antihistamínicos sintéticos ha dado resultado excelente en lapsos entre 30 y 80 minutos, aun cuando existía compromiso general.

No tuvimos oportunidad de ensayar otras terapéuticas.

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19. TOXIC ACTION OF ROVE BEETLES (*COLEOPTERA, STAPHYLINIDAE*)

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Introduction

The vesicant action of beetles belonging to the genus *Paederus* (STAPHYLINIDAE) was recognized for the first time by da Silva, in 1912 (1), in Brazil.

The species involved was *Paederus columbinus* Lap.. This insect is frequently encountered in the region of Bahia from June to September, especially along the borders of the main streams. It appears to be a positive pest for people working in the fields of potatoes, corn or beans and it is well known to them under the name of "potó" or "trepa moleque". When these insects alight on the bare skin they produce a pruriginous erythema which is followed by a blister and a slowly healing ulceration. These lesions occur on the legs, the arms and the neck.

Since the paper of da Silva (1) many other authors have reported similar observations in different parts of the world and involving various species of *Paederus*. A general review of that question appeared in the thesis of Allard (2), in 1948. This work was completed by Theodorides in 1950 (3) and more recently by the thesis of Gruvel (4). Several other contributions have been added during the last years. Among them the most important seem to be those of Pavan and Bo (5, 6) and Pavan (7, 8) on the nature of the toxic factor responsible for the cutaneous or ocular lesions.

The rove beetles are characterized by their narrow body and their short elytra under which the long membranous posterior wings are folded. They have beautiful colours. In *P. sabaeus* the head and the posterior segments of the abdomen are black, the thorax and the anterior part of the abdomen brownish-yellow, the elytra metallic blue. Most of the species are small, measuring not more than 1 cm in length. Some species have the habit of curling the abdomen over the thorax, and they may therefore easily be mistaken for flying ants.

Geographical distribution of the rove beetle dermatitis

The dermatitis produced by the beetles of the family STAPHYLINIDAE has been observed on the five continents. So far the vesicant properties have been recognized as belonging only to the genus *Paederus*. It is to be noted, however, that the species *ruficollis* which is known as producing experimental dermatitis, has been removed recently from the genus *Paederus* and placed

in the genus *Paederidus* Rey (Fig. 6). According to Mr. G. Fagel of Brussels, the well-known specialist in the study of these beetles, some of the South-American species could, in fact, belong to other genera of the STAPHYLINIDAE. The genus *Paederus* has a world wide distribution and is represented by several hundred species among which a small number, not more than 30, have been recorded in relation with human dermatitis. It seems, however, very probable that the majority of the species of *Paederus* contain a vesicant fluid. Specific identification in that group is very difficult and may be made only by an expert.

In South America the rove beetle dermatitis has been reported for the first time in Bahia, Brazil, by da Silva in 1912 (1) in connection with *Paederus columbinus* Laporte.

Soon thereafter, Göldi in 1913 (9) observed the same toxic action in the species *Paederus goeldi* (Wasmann, 1905) in Amazonas, Brazil. A third species, *Paederus amazonicus* (Sharp) has been mentioned by Bequaert (10) as causing dermatitis, in the same country. Another recorded from Brazil is that of Gordon (11) who stated that *Paederus amazonicus* had been first encountered at Manaus, Amazonas, in January 1921. Froes (12, 13) in Bahia, Brazil, showed experimentally that *Paederus brasiliensis* Er. (Fig. 5) and *Paederus rutilicornis* Er. are able to produce vesicular dermatitis. More recently Pickel (14) has mentioned the presence in Brazil of 20 species of *Paederus*, among which only 6 are strongly vesicant. He also noted that *Paederus ferus* Er. and *P. brasiliensis* Er. produce dermatitis in Pernambuco.

In Ecuador epidemic vesicular dermatitis in man was observed by Chapin (15) in 1926 in connection with *Paederus irritans* Chap.. Soon after, Campos (16) reported cases produced by 8 different species of *Paederus*. Earle (17), in 1949 described cases of dermatitis in connection with *Paederus ornaticornis* Sharp. In this country the disease is known under the name "Fuetazo dermatitis". The name "fuetazo" is a spanish word for whiplash. It calls attention to the linear aspect of the lesions, especially when they occur on the face.

According to Dallas (18, 19) vesicular dermatitis is produced in Argentina by *Paederus brasiliensis* Er. and *P. ferus* Er..

Bequaert (20) has shown that *Paederus signaticornis* Sharp causes dermatitis in Guatemala .

In Africa the first report of rove beetles dermatitis is that of Rodhain and Houssian in 1915 (21) in Leopoldville, Congo. The species involved in these cases was identified by H. Notman (in Bequaert, 10) as *Paederus sabaeus* Er. (Fig. 3). In 1916, Ross (22) showed that *Paederus eximius* Reiche (synonym = *P. crebrepunctatus* Eppelsheim) is the agent of a vesicular dermatitis in Kenya (Fig. 2). In several parts of Kenya, especially in Nairobi, this species may also produce a severe conjunctivitis which is known as "Nairobi-Eye" (see Symes and Roberts — 23 — and Roberts and Tonking — 24 —). Other records of dermatitis produced in Africa by *Paederus sabaeus* are those of Gordon (11) in West Africa, of Lewis (25) in Sudan and of Denys and Zumpt (26) in South West Africa and in Nyasaland.

In Europe, Sacharow, in 1915 (27) reported cases of dermatitis produced by *Paederus fuscipes* Curt (= *P. idae* Sharp) in Southern Russia (Fig. 1). Several other authors have confirmed these observations (Portchinsky, 1915 — 28 —, and Pawlowsky and Stein, 1926 — 29 —). This species is very common along the banks of the lower Volga and fishermen and herdsmen are commonly

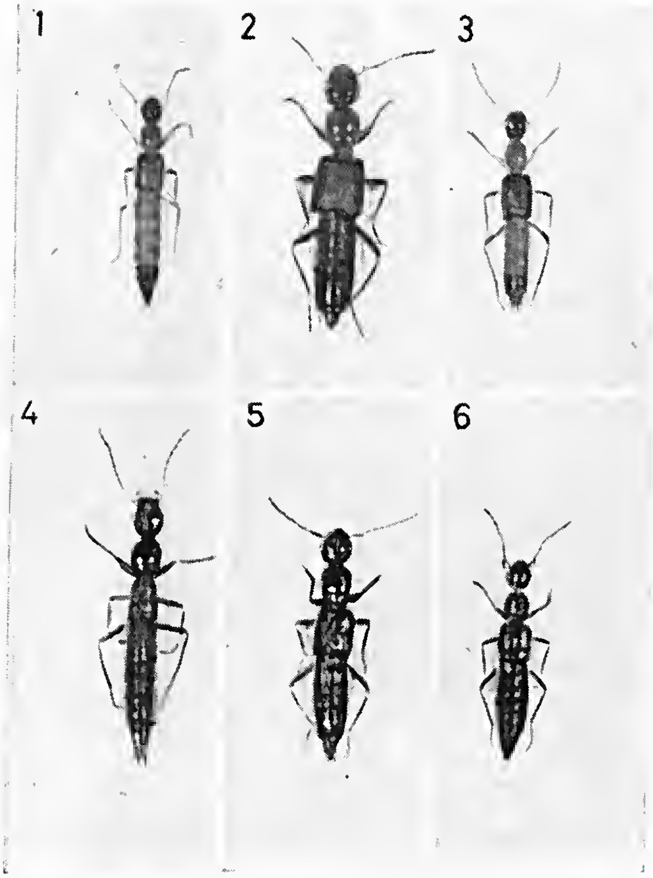

Fig. 1-6 — Some harmful Rove-beetles:

Fig. 1 — *Paederus fuscipes* Curtis
Fig. 2 — *Paederus eximius* Reiche.
Fig. 3 — *Paederus sabaeus* Er.
Fig. 4 — *Paederus ornaticornis* Sharp
Fig. 5 — *Paederus brasiliensis* Er.
Fig. 6 — *Paederidus ruficollis* Fabr.

(Photograph made by Institut des Sciences Naturelles de Belgique, Brussels).

blistered by the crushing of beetles on the skin. In 1919, Netolitzky (30), in Bukovine, was able to produce experimental dermatitis in man with two other European species (*Paederus limnophilus* Er. and *Paederus ruficollis* Fab. — Fig. 6). Experimental dermatitis in man has also been produced in France by Allard (2) with *Paederus littoralis* Grav. and by Theodorides (3) with *P. riparius* L. and *P. fuscipes* Curt.. It is in Italy that the irritant action of *Paederus* on the eye has been recognized for the first time by Farina in 1926 (31). These lesions seem to be rather common in that country and Castelli in 1935 (32) recorded numerous cases of conjunctivitis that he called "Oftalmozoosi endemica di *Paederus*". All these ocular lesions were produced by *Paederus fuscipes*.

In Asia the vesicular dermatitis has been reported from several countries. In India the disease is known as "Spiderlick" and is produced mainly by *Paederus fuscipes* Curt. (cited by Strickland — 33 — Strickland and Roy — 34 — and Isaac — 35 —). Pujatti, in 1947 (36) reports that *Paederus melampus* Er. produces dermatitis in the State of Benagolore (India).

According to Eysell (37), *Paederus peregrinus* Er. is able to produce similar lesions in Malaysia.

In Indochina epidemic dermatitis is produced by *Paederus fuscipes* and *P. alternans* Walk (see Genevray and col., 38).

Rove beetles dermatitis has also been observed in Japan by Wada in 1926(39). The species involved was *Paederus riparius* L.. Esaki (40) reports that the dermatitis produced by *P. idae*, which is a synonym of *P. fuscipes* Curt. has been known in Japan, Korea and China since 1590, and Kandu Kanji, in 1935 (41), mentions that severe ocular lesions are produced by this species in Formosa.

More recently the vesicular dermatitis has been reported from Australia, by Millard in 1954 (42). The disease is produced by *Paederus cruenticollis* Germ.

BIOLOGY OF THE *PAEDERUS* SPP.

Many of the authors observing rove beetle dermatitis in man have drawn attention to the seasonal character of the disease. In Brazil *Paederus columbinus* is encountered from June to September. In Ecuador the beetles appear with the first rains in December or January and persist until the onset of the dry season in May or June. In Leopoldville, Central Africa, *Paederus sabaeus* is frequent from April to June, and completely absent at any other time of the year (Rodhain and Houssiau and personal observations). This period corresponds with the end of the raining season and the beginning of the dry season. According to Dr. C. Rossetti (University Lovanium), the beetle population in Leopoldville reaches a peak every two years (personal comm.). In South West Africa, Deneys and Zumpt (26) report cases of dermatitis produced by *P. sabaeus* during the month of December. In Freetown, Sierra Leone, *P. sabaeus* is fairly common during June, July and August; it disappears during September and October but reappears in the middle of November (Gordon, 11).

In India, Isaac (35) has noted that *Paederus fuscipes* reaches its greatest density in May and June, when the vesicular dermatitis become abundant. In the same country Pujatti (36) made similar observations with *Paederus melampus* which occured mainly from May to July.

These observations show that in tropical regions the beetles are most frequent or at least most active during the rainy and hot season. That a high degree of humidity is an important condition for the activity of these beetles is shown by the fact that *Paederus fuscipes, P. sabaeus, P. eximius* and other species are always found close to river banks or swampy areas. In Europe the most favourable conditions are realized at the end of the Summer.

This periodicity seems, however, not to be absolute, for in Manaus, Amazonas (Brazil), Gordon (11) reports that *Paederus amazonicus* is common in all the seasons of the year.

Another feature that is to be mentioned in relation with the biology of those beetles is the fact that some speeies are attracted to the artificial light. It seems therefore very probable that some cases of dermatitis are contracted during the night.

Pathogeny of the rove beetles

Dermatitis is the most commonly pathological feature observed in relation with rove beetles; however, in some eases eye lesions may also have been produced. The latter are generally a resnlt of the spread of the irritant with the fingers and they are therefore secundary to the crushing of the insect on the skin.

The skin lesions consist essentially of a vesienlar dermatitis and they are usually seen on the exposed parts of the body. The elinical evolution of this dermatitis is rather characteristie. A good description has been made by Theodorides (3) who experimented upon himself with *Paederus fuscipes*. This author divided the lesions into three stages: an *erythematous stage* characterized by a red pateh, a *vesicular stage* following the preeeding one during whieh the blisters appeared, and a *squamous* or *healing stage*. The erythematous stage developed approximately 10 hours after contact with the beetle and it persisted during 48 hours. The maeula was not spontaneonsly painful but presented a tickling or burning sensation. The first blisters appeared 48 hours after the beginning of the erythema. They enlarged gradually and attained their complete development within 2 days. During the healing stage the vesicules became umbilieate and dried out. Finally they exfoliated leaving pigmented sears which persisted for two months. The healing stage began 6 days after the eontact with the beetle; it persisted for about 8 days.

The spontaneous dermatitis that I have observed in Leopoldville with *Paederus sabaeus* presents a similar evolution (Fig. 7). In some cases the maeulas coalesee into large areas up to 10 cm in diameter. A linear dermatitis, 5-15 em long, may appear at the place where the beetle has been dragged along the skin when brushed off by the hand. The lesions are not spontaneously painful;

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Fig. 7 — Rove-beetle dermatitis on the arm of an African in Leopoldville. (Photograph made by Prof. C. Rossetti).

however, a slight burning sensation followed by itching may be present. The vesicules, which are generally very small, contain either a clear or a seropurulent fluid. In some cases they coalesce to form a single large blister. The lesions produced in East Africa by *Paederus eximius* (= *P. crebrepunctatus*) are more severe and are often accompanied by general symptoms.

When the irritant enters into the eye, conjunctivitis may occur. Ocular lesions have been described for the first time in Italy. They were produced by *P. fuscipes*. These lesions are more frequently encountered and more severe with *Paederus eximius* than with other species of *Paederus*. They are well known in Kenya as "Nairobi-Eye".

According to Göldi (9) the hemorrhagic enteritis, well known in the Marshall Islands as "Toddy Disease" is probably produced by swallowing of palm-wine (called also "toddy") into which some rove beetles have fallen. So far the toxic action of rove beetles for the digestive tract in man or in animals is not established with certainty. In that respect it seems that these beetles are less pathogenic than those of the family MELOIDAE whose irritating power for the digestive mucosa is well known in man and in animals.

Nature of the irritant principle of the rove beetles

Da Silva (1) thought that the irritant substance responsible for the dermatitis was a secretion of the beetle.

Rodhain and Houssiau (21) experimenting on man with *Paederus sabaeus* in Leopoldville showed that when the insects were allowed to wander freely over the skin and even when they were irritated, no lesions, either immediate or delayed, were produced. The lesions appeared only when the beetles were crushed and rubbed over the skin. Other authors have repeated these experiments with the same results.

Gordon (11) found that the reaction occurs on areas smeared with the thorax and the abdomen of the beetle but none with the head.

Pawlowsky and Stein (43), experimenting with *P. fuscipes*, have shown that the irritant principle is always present in the genital organs and that it reaches its highest concentration in the haemolymph.

Some observations have proved that the irritant can be absorved through a non-injured epidermis. It seems probable that the softening of the skin by an excessive perspiration or after bathing makes this penetration more easy and that on the contrary a greasy condition of the skin lessens its action.

So far it had been commonly thought that this irritant was cantharidin. Netolitsky (30) was the first to surmise that the active principle contained in the rove beetles is not cantharidin. Pawlowsky and Stein, in 1920 expressed the same opinion. The true nature of this principle has been shown by Pavan and Bo (5, 6) and by Pavan (7, 8). These authors succeeded in isolating and obtaining in its pure crystalline state the active principle of the vesicant substance, to which they give the name of "pederin". They also proved that pederin is clearly distinct from cantharidin not only in terms of its biological but also in terms of its physical and chemical properties.

Diagnosis of the rove beetle dermatitis

The diagnosis of the disease is generally not difficult. It should be based on the following characteristics:

1. The sudden appearance of the lesions.

2. The absence of bilateral symmetry of the lesions. Generally only one region of the body is affected.

3. The aspect of the lesions consisting often of a group of small vesicles or of a single larger vesicle. The linear grouping of the vesicles is characteristic.

4. The seasonal and epidemic character of the disease.

The long delay (2 to 3 days) between the contact with the beetle and the appearance of the vesicles may render the diagnosis difficult in absence of an epidemy.

It may be difficult to distinguish the rove beetle dermatitis from the cantharid dermatitis which is produced by beetles of the family MELOIDAE, especially the genera *Lytta*, *Epicauta* and *Mylabris*. The best known of them is the bright metallic green "Spanish fly", *Lytta vesicatoria*.

According to Theodorides(44) the vesicle produced by cantharidin develops on the normal skin while that produced by the *Paederus sp.* is always preceded by an erythema.

Bo and Vancurone (45) have summarized in a table their experiences on man with either cantharidin and pederin (see Table I).

TABLE I — DIFFERENCES BETWEEN THE EXPERIMENTAL DERMATITIS PRODUCED IN MAN BY CANTHARIDIN AND THAT PRODUCED BY PEDERIN. (AFTER BO AND VALCURONE, 1958).

	Lesion produced by catharidin (beetles of the family MELOIDAE)	Lesion produced by pederin (beetles of the family STAPHYLINIDAE)
Delay between the contact of the irritant and the onset of the erythema	18 — 24 hours.	36 — 72 hours.
Character of the erythema	Mild, without any subjective symptoms.	Well developed and painful.
Character of the vesicle	Small bullae coalescing quickly into a single voluminous blister. This blister contains a clear fluid and is situated on a superficial base. Subjective symptoms almost absent.	Small, or very small, vesicles containing clear fluid transforming into larger pustules (with purulent fluid) which tend to coalesce. These pustules are situated on a deep base. Subjective symptoms, very marked, consisting of pruritis and burning sensation.
Healing	Either by resorption or bursting of the blister.	By formation of a squamous crust.
Residual lesion ...	Pigmentation, if present, is light and disappears rapidly.	Pigmentation well developed and persisting. Pruritis may be present.

Treatment of the rove beetle dermatitis

Roberts and Tonking (46) have treated the blisters with Magnesium Sulfate compresses.

Earle (17) recommends a formula containing, among other drugs, Butesin picrate.

Deneys and Zumpt (26) found that cortisone and antihistaminic preparations had no beneficial effect and may even be harmful by favouring secondary infection. They recommend to treat the more severe lesions by protection and the application of an antibiotic powder.

In Leopoldville, on the advice of Professor C. Rossetti (University Lovanium), we have treated our cases with the water paster of Darier (equal parts of Talc, Zinc Oxyde, Glycerine, Water) with good results.

Acknowledgments — I am indebted to Prof. C. Rossetti, University Lovanium, Leopoldville, Republique of Congo, who furnished unpublished information and photograph on rove-beetle dermatitis at Leopoldville.

I also wish to thank Mr. G. Fagel, Institut des Parcs Nationaux du Congo, Brussels, who reviewed the taxonomic part of the manuscript and supplied the material for illustration of some *Paederus* sp.

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20. METRONIDAZOLE IN SNAKE VENOM POISONING

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The use of metronidazole [1(β-hydroxymethyl)-2-methyl-5-nitroimidazole] in the treatment of 43 patients having 67 indolent, vascular lesions of diverse origins has been noted by Taylor (1). In her series of cases, improvement of the lesions as demonstrated by decreased vasculitis and perivascular infiltration was noted in 42 patients (63 lesions). One of these patients was bitten by an adult prairie rattlesnake, *Crotalus horridus*, and treated initially by the present author. Following hospitalization for the acute stages of the poisoning, the patient was referred to Dr. Taylor for metronidazole therapy. Four additional patients were subsequently treated with this drug following the acute stages of their envenomations. The present report treats of the observations on three of these patients.

Case 1 — A 12-year-old boy was bitten on the right middle finger by a 134 cm Southern Pacific rattlesnake *Crotalus viridis helleri*. The finger was immediately placed in ice and the patient taken to a hospital. He was given one vial of Antivenin (Polyvalent) CROTALIDAE and an intramuscular corticosteroid. The finger was kept in ice for five days. On the sixth day, the ice was discontinued and the patient transferred to a second hospital for possible amputation of the injured finger (Fig. 1). The author was called on consultation.

Under anesthesia, the necrotic areas were surgically debrided. A program of physical therapy was initiated on day seven, and the patient placed on metronidazole, 100 mg four times daily for eight days. Healing appeared to be more rapid than generally expected in such cases and the patient was discharged from the hospital 16 days following the accident. A slight contracture of the finger was subsequently corrected. Figure 2 shows the finger two months following the bite.

Case 2 — A 9-year-old boy was bitten on the left foot in the region of the medial malleolus by a large red diamond rattlesnake *Crotalus ruber ruber*. Longitudinal cuts were made through the fang marks approximately 45 minutes after the bite. Suction was not applied. Because of the child's known sensitivity to horse serum, he was referred to the author for further medical care.

The patient was first seen by the author eight hours following the accident (Fig. 3). In spite of the lapse of time, 2.1 gm of *C. ruber ruber* antivenin, prepared in goats after the method of Criley (2), was injected intravenously over a 30-minute period. Further therapeutic measures, as previously described (3), were carried out. At day three the skin lesions extended to the knee (Fig. 4).

Fig. 1

Fig. 2

On day four the hemorrhagic vesicles and necrosis were debrided by surgical excision under anesthesia. On day five physical therapy was initiated, and the patient placed on metronidazole, 125 mg four times daily; this was continued

Fig. 3

Fig. 4

for one week. The patient was discharged from the hospital 10 days following the accident. There was no residual. Figure 5 shows the foot one month following the bite.

Case 3 — A 44-year-old reptile handler was bitten on the dorsum of the left thumb by an extremely large (170 cm) timber rattlesnake *Crotalus horridus horridus*. It was suspected that one fang pierced a blood vessel as there was excessive bleeding from one of the puncture wounds. The patient was rushed to the hospital, arriving in a cyanotic and comatose state (B.P. 50/0). Emergency measures consisted of fresh whole blood, intravenous antivenin (including poly-

Fig. 5

Fig. 6

Fig. 7

Fig. 8

valent *Crotalus* antivenin prepared in goats), oxygen vasopressor agents, localized infiltration of the wounds with calcium disodium edetate (EDTA) and measures previously described (3). The patient's condition remained critical for four days, during which time he received 10 pints of blood.

Figure 6 shows the left hand and forearm four days following the envenomation. At day seven the vesicles and necrotic areas were surgically debrided under anesthesia (Fig. 7), and physical therapy was instituted. Subsequently, the patient was placed on metronidazole, 250 mg four times daily for two weeks. Healing appeared to be rapid (Fig. 8).

An infection over the base of the first metacarpal phalangeal joint necessitated surgical drainage. The incision exposed the extensor tendons and bone, and a subsequent chronic osteomyelitis of the joint developed. The complication lead to some loss of bone and soft tissues. Orthopedic surgery corrected most of the defect so that the patient now has some use of the thumb.

The lesions produced by the venoms of most of the North American rattlesnakes are troublesome in that they are usually multiple and often times large, and thus particularly susceptible to infection. They are also painful and very slow to heal. The present limited series of cases indicates that the use of metronidazole following the acute stages of the poisoning should be given further clinical trial. The drug appeared to increase healing and perhaps reduce pain over that expected in similar cases of envenomation by rattlesnakes.

There would not seem to be any contraindication for the use of the drug, although administration should be limited to the period following the acute stages of the poisoning, which generally persists for 4 to 6 days following the bite. It should not be used in the presence of serious systemic poisoning.

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21. THE RELEASE OF BIOGENIC AMINES FROM BLOOD PLATELETS UNDER THE INFLUENCE OF *CROTALUS D. TERRIFICUS* VENOM

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In previous studies on the action of certain snake venoms and of bee venom on blood platelets we found, that the venom of the Brazilian rattlesnake (*Crotalus d. terrificus*) causes in even very small concentrations a release of biogenic amines and of ATP from these cells (1). The venom component responsible for this effect is of special interest with regard to an analysis of both the action of the venom and the mechanism of amine release. Therefore, we endeavoured in the isolation and characterization of the amine releasing component of the venom and in clarifying its mechanism of action.

In order to isolate the active amine releasing principle, *Crotalus* venom was disassembled into several components by gelfiltration on columns of Sephadex G 100. The experimental results are summarized in Fig. 1. A separation of the amine releasing component from clotting and phospholipase activities and other ballast proteins could be established. The fractions active on blood platelets were collected and freed from buffer salts by dialysis against destilled water. The specific activity of the solution obtained was 20 times higher than that of the crude venom measured by the serotonin release. The yield of active substance averaged 60-65 per cent. The factor gives the reactions of a high molecular protein.

Furthermore it was studied, whether the amine releasing component posseses other biochemical activities, known to be present in the whole venom, besides its action on blood platelets. It was shown, that the isolated component is related to one of the activities, which could be demonstrated and investigated with whole venom, with the exception of only a small caseinolytic activity. Moreover, pharmacological reactions caused by whole venom as well as general toxicity were found not to be connected with it.

Serotonin release induced by the amine liberating component proceeds with high speed. Under the experimental conditions used more than 50 per cent of the stored amines were released during 5 min by only 0,01 μg of the isolated component per ml. Amine liberation by the venom factor can be inhibited by omitting of calcium and by blocking of the platelet metabolism (Fig. 2). After incubation of platelets with the amine releasing component in the absence of calcium the cells release their serotonin already by resuspending them in a medium containing calcium. Amine liberation by the active venom component is also possible in blood. In the presence of plasma its effectiveness is only slightly diminished. Therefore, we also investigated the action of the isolated component in the circulating blood by injecting it intravenously into rabbits under urethan narcosis. Intravenous injection of the component was followed

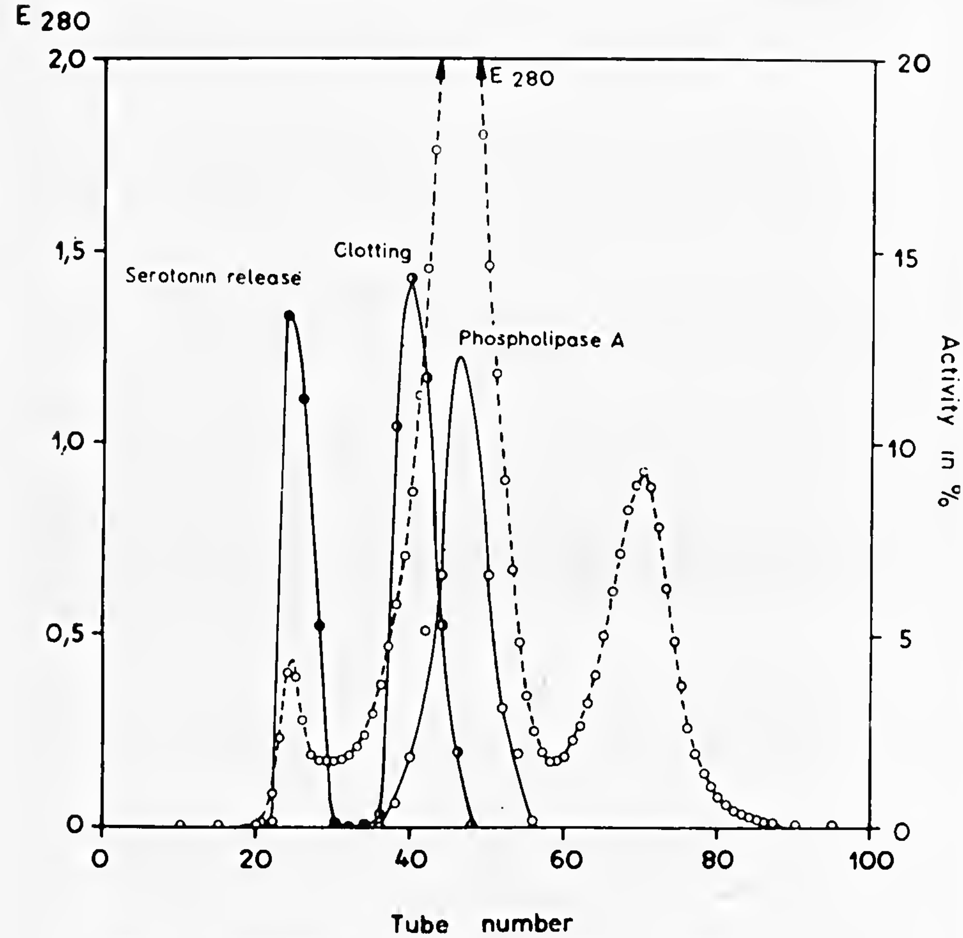

Fig. 1 — Gelfiltration of *C. d. terrificus* venom on Sephadex G 100.

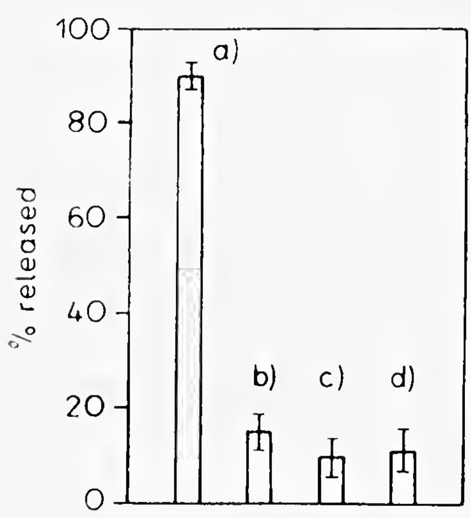

Fig. 2 — Release of serotonin from rabbit platelets by *C. d. terrificus* venom. a) in Tyrode-solution; b) without glucose; c) with 10^{-3} m monoiodoacetic acid; d) with EDTA.

by a fall of the blood pressure, which in the same intensity could be produced also by injection of a serotonin quantity equivalent to the amount contained in blood platelets. A second injection did not result in a blood pressure response (Fig. 3). The number of thrombocytes was found to be lowered to 50 per cent after the injection. Platelets isolated from the blood after administration of the venom factor contained only 20 per cent of its original serotonin level.

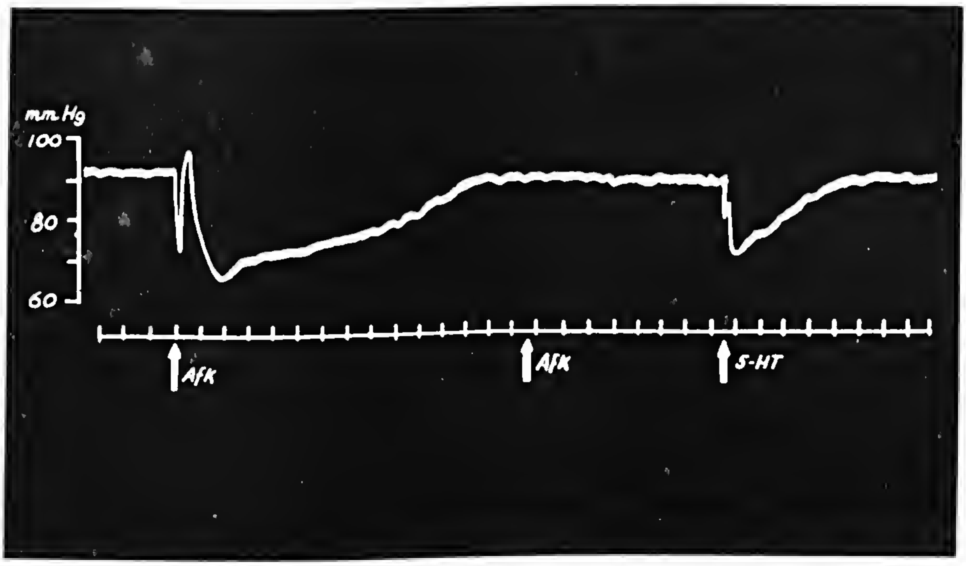

Fig. 3 — Effect of the isolated amine liberating component of *C. d. terrificus* venom (AfK) on blood pressure.

Conclusion — In the venom of the Brazilian rattlesnake (*Crotalus d. terrificus*) there exists a relatively high molecular protein component, which is able to liberate stored biogenic amines from blood platelets. The factor is active in platelet suspensions *in vitro* as well as in the circulating blood. This component is not identical with other biochemical and pharmacological active principles of the whole venom. The induced release-reaction seems to proceed in two steps. In the first step the amine releasing venom factor induces a change in the permeability of the platelet membrane, and in a subsequent reaction amines became liberated.

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22. FURTHER OBSERVATIONS ON CORAL SNAKE BITES IN THE UNITED STATES: SYMPTOMS AND THERAPY

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Since our first observations on the bite of a North American coral snake were published (*Journal of the Florida Medical Assoc.*, **49**, 968, 1963), our work has continued along parallel experimental and clinical lines. The snakes used in these experiments are part of a colony of over two hundred animals, primarily of *Micrurus fulvius*, from which venom has been collected for toxicological study.

Histological studies of the venom apparatus of the North American coral snake indicate that the venom gland is a combined serous and mucus secreting organ. The mucus portion of the gland is somewhat more extensive in proportion to the total glandular structure than that which has been described in the pit viper (Gennaro *et al.*, New York Academy of Sciences) and other vipers (Kochva & Gans, American Journal of Anatomy). Since it has been shown that the mucus secreting portion may be responsible for spreading and enhancing the toxicity of the serous secretion, the increase in relative size of the mucus secreting portion of the gland may be responsible, in the coral snake, for the high level of toxicity manifested by the venom.

The lyophilized venom of *Micrurus fulvius* used in the experiments here was titer for lethality in mice and dogs. The $L.D._{50}$ in the albino mouse (20 grams) is 0.23 microgram per gram of mouse or 0.23 milligrams per kilo. The $L.D._{50}$ value in dogs is 0.3 microgram per gram or 0.3 milligrams per kilo. Though these values appear to be similar, it is easy to see that the amount of venom injected in the dog is really much greater than that lethal in a mouse. It is interesting to note that the dog exhibited very little variation in sensitivity to this venom as they commonly do to the venom of the *Crotalus* snakes.

The symptoms most frequently occurring in coral snake envenomation in dogs followed two general patterns.

In sublethal doses the initial aspect (first hour) was characterized by a drowsiness and favoring of the injected leg. The total course of envenomation in the sublethally injected dog proceeded with various body tremors, some respiratory difficulty, and dizziness .possibly associated with bloody urine.

In lethally injected animals the course of symptoms presenting in the first hour included vomiting and a general parasympathetic-stimulation picture which included salivation and defecation. Death in these animals usually followed paralysis, respiratory difficulty, continued salivation, and repeated vomiting, some impairment of the blink reflex, and fixation of the pupils centrally. No retinal hemorrhage is indicated although hematuria is common and blood can appear from most of the body orifices.

In cases of actual snake bite the character of the bite itself, and its duration, was established to be more painful than has been recorded in the literature and much more quickly occurring. Some pain, favoring, and tenderness always seem to accompany the bite which lasts from four or five seconds to too short a period to measure using a stop watch; all bites of sufficient duration do produce a lethal effect.

In most of the human cases recorded in the literature death occurs within twenty-four hours or not at all. In dogs, the $L.D._{50}$ dose produces survival in the range of 9½ hours. When this dose is increased by five times the survival is shortened but there is considerable variation in the survival period which can be as little as fifty-eight minutes or as long as five and one-half hours at this dose.

The $L.D._{50}$ dose is completely reversible by the administration of antiserum (Butantan anticoral or the rabbit anticoral made in our laboratory). The first effect of the antiserum is to lengthen the survival time. Doses as high as three times the $L.D._{50}$ may be survived if the antiserum is given in a dose sufficient to provide 2½ milliliters for every milligram of venom injected as soon as two hours after the bite (intravenously). The importance of the treatment time is illustrated by the fact that 3.3 milliliters of antiserum per milligram of venom is valueless if the treatment time is delayed until five hours after the bite. On the other hand the $L.D._{50}$ dose may be neutralized and the animal may survive with only 1.4 ml of antiserum per milligram of venom if it is given within the hour after the bite.

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23. INYECCIÓN *IN SITU* DE ANTISUERO EN EL TRATAMIENTO DE LA INTOXICACIÓN POR MORDEDURA DE SERPIENTE

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Presento ante la ilustrada consideración de ustedes un procedimiento senoillo, pero lógico, para neutralizar el veneno de serpiente en el mismo lugar en donde lo depositó el animal al producir la mordedura.

El veneno de las serpientes, al ser inoculado, causa síntomas graves: generales tóxicos y locales en el mismo lugar de la mordedura.

La acción local sobre los tejidos se ejerce por medio de enzimas. Tiene una rápida acción difusora por la hialuronidasa que contiene, que hidrolisa el ácido hialurónico del tejido conjuntivo.

Además de esta acción de difusión, el veneno, principalmente el de *Bothrops*, produce lesiones en los vasos sanguíneos, alterando las células endoteliales y provocando la disolución de sus paredes, por medio de lecitinasas y proteinasas. Consecuentemente, provoca extravasación de eritrocitos hemolisados e de suero en los tejidos. El resultado es un edema hemorrágico muy extenso.

Las propiedades digestivas de los venenos, más acentuadas en el de las *Bothrops*, unido a la acción destructiva sobre los vasos sanguíneos, provoca grandes necrosis de los tejidos, a tal grado, que estos llegan a desprenderse, dejando los huesos al descubierto.

Se atribuye a las reacciones enzimáticas la producción del choque. Y se ha achacado a la histamina el descenso de la presión sanguínea, histamina que ha sido liberada en las lesiones celulares causadas por la lecitinasa.

Como es tan sabido, desde 1897, los sueros se producen usandose como antígenos venenos de serpientes *Bothrops*, *Crotalus*, etc. que provocan la formación de anticuerpos. Los anticuerpos se caracterizan por su acción electiva sobre los antígenos que intervinieron en su producción. Para que los anticuerpos puedan ejercer su acción neutralizante, deben ponerse en contacto con el antígeno homólogo.

En la titulación del antiveneno se hacen mezclas de antiveneno y veneno, para determinar la dosis de veneno que es neutralizada por un ml de suero.

El problema — Neutralizar la acción tóxica del veneno, antes de que sea absorbido por el organismo.

Cuando ocurre un accidente ofídico, la primera preocupación que se tiene, es la eliminación del veneno, para lo cual se ha recurrido hasta a la amputación del miembro lesionado. Pero lo que más se usa es la desbridación amplia del sitio mordido, para succionar el veneno, sea con la boca directamente o mediante

el uso de ventosas. Procedimiento que tiene inconvenientes por provocar hemorragias e infecciones y la fácil contaminación de la herida por el *Clostridium tetani.*

El antisuero homólogo, en inyección intramuscular, neutraliza al veneno ya absorbido que se encuentra en la sangre circulante. Pero no llega a evitar las graves lesiones locales que el veneno a una alta concentración en los tejidos, produce mediante sus enzimas.

El problema es eliminar la acción local y la general del veneno, tal como se ha pretendido por la amputación y la desbridación con succión.

Resolución — Antes hemos mencionado que el suero se titula según la dosis de veneno que neutraliza un ml "in vitro", por lo tanto, se supone que también pueda ser neutralizado el veneno por el antisuero homólogo en el mismo sitio en el que la serpiente lo depositó, en los tejidos de la víctima.

Bajo estas premisas procedimos a la experimentación.

Experimentación

Veneno empleado — Veneno deshidratado por medio de liofilización de *Bothrops atrox*, en solución salina al 3% (cada mililitro corresponde a 30 mg de veneno).

Suero — Proporcionado por los Laboratorios "MYN", S.A. de México, con título neutralizante de 12.5 mg de veneno deshidratado por un mililitro.

Animal usado — Conejos de aproximadamente 3 kg. de peso corporal.

Lugar de inoculación del veneno — Parte superior de uno de los muslos, previamente depilado.

Vía usada — Subcutánea.

Se emplearon 3 grupos de 5 animales cada uno:

Al grupo A se le inyectó un mililitro de la solución (30 mg de veneno).

Al grupo B se le inyectó la misma dosis de veneno y en seguida se le inyectó 4 ml de suero Antibothropico, por vía intramuscular, en el otro muslo.

Al grupo C se inyectó con la misma dosis de veneno (1 ml = 30 mg) e inmediatamente después 4 ml de suero "in situ", de tal manera que se mezclaron.

Resultados

Los conejos del grupo A, a quienes solamente se les inyectó el veneno, murieron todos en un promedio de una hora y media después.

Los conejos del grupo B, a quienes además del veneno recibieron 4 ml de suero por vía intramuscular, sobrevivieron, pero se les produjo, en el lugar de la inyección del veneno, un extenso edema, a las 24 horas, con formación de escara a las 48 hs. Los conejos del grupo C, a quienes se les inyectó el suero en el mismo lugar de la inyección del veneno, solamente se observó, en el lugar de la inyección, un pequeño edema que desapareció después de 4 días. No se formó escara.

Técnica de la inyección del antisuero — Deve hacerse en la región mordida una verdadera infiltración del antisuero de tal manera que aún que el veneno haya emigrado a la parte declive del miembro lesionado, llegue a mezclarse con el veneno y lo neutralice.

Los conejos que recibieron la inyección del antisuero por vía intramuscular y sobrevivieron, los del grupo B, la escara no se formó precisamente en el lugar de la inyección del veneno, sino en la parte declive del muslo. Tal vez la acción difusora del veneno al hidrolizar el ácido hialurónico del tejido conjuntivo facilita su migración. Por lo tanto debe tomarse en cuenta esta mobilización del veneno para la inyección del antisuero, de modo que lleguen a mezclarse.

Caso clínico — En la práctica hemos tenido la ocasión de observar a uno de nuestros empleados quien tuvo tres accidentes por mordedura de *Crotalus d. terrificus*. En el primer accidente se le inyectó el suero "in situ" y además, por vía intramuscular, una dosis de suero. Localmente no presentó más tarde ninguna lesión, y los síntomas generales fueron sin importancia. En la zona mordida solamente se observó ligero edema que desapareció a las 48 horas.

El segundo accidente consistió en una mordida en el índice izquierdo y fué una lesión superficial, por tal motivo y tomando en consideración que debía de haber una sensibilización al suero y a petición de la víctima, no se le inyectó suero. Después de 2 horas, se tuvo que inyectar de emergencia debido a los graves síntomas de intoxicación que se desencadenaron. Salvó la vida, pero la lesión que se produjo en el índice tardó en cicatrizar más de un mes con peligro de perder la falangeta.

El tercer accidente fué la mordedura en el dorso de la mano derecha por una *Crotalus*. Inmediatamente se procedió a inyectarle "in situ" el suero homólogo (Anticrotálico). En esta ocasión no se produjo ninguna acción destructiva y no se presentaron síntomas de intoxicación ya que además recibió una inyección intramuscular de suero.

Condición "sine qua non" — Para que este procedimiento de neutralización del veneno por el antisuero sea útil, debe aplicarse tan pronto como se produce el accidente ofídico. Con tal objeto debe encontrarse el suero allí en donde precisamente se necesita, acompañado de una jeringa estéril y algodón con un antiséptico. Ciertamente que el suero líquido tolera por un año la temperatura ambiente, pero pierde el 50% de su actividad durante un año a 30°C. En cambio el suero liofilizado tolera esa temperatura durante un máximo de 5 años. El suero cuyas proteinas han sido modificadas por digestión enzimática conserva su actividad por más tiempo que el suero líquido.

El suero liofilizado debe formar parte del botiquín de emergencia que deben usar nuestros campesinos. Además debe capacitarse a las personas responsables para aplicar el suero inmediatamente. De tal manera que de una tremenda emergencia como es la mordedura de serpientes venenosas pasa a ser un accidente sin importancia con sólo inyectar "in situ" el suero homólogo. Ya es tiempo de evitar la muerte de nuestros hombres del campo por mordedura de serpiente. Cuando la víctima es transportada al hospital más cercano, o fallece en el camino o si se salva sufre la acción local del veneno, con la formación de esfacelos, que tardan varios meses para cicatrizar y que muchas veces terminan por amputación del miembro lesionado, después de permanecer hospi-

talizado bastante tiempo. Hospitalización que además de ser costosa impide al enfermo trabajar y por lo tanto deja de producir, para más tarde convertir-se en un liciado.

Este procedimiento que, como una comunicación previa fué publicado en la Revista Mexicana de Ciencias Médicas y Biológicas en el número correspondiente a los meses de enero-febrero de 1952, lo presento ahora ante la docta consideración de ustedes como un homenaje a Vital Brazil, el fundador no sólo del Instituto Butantan, sino de la seroterapia antiofídica de nuestras Américas.

Summary

The venom of *Bothrops* snakes, generally produce local necrosis in the tissues, due to its intense proteolytic action. In this paper an injection method "in situ" of the homologous antiserum was proposed, to avoid those lesions, neutralizing the venom at the same site of the bite, thus preventing for the most part the general and local activities of the venom.

The results are effective to such extend, that the employement of this procedures is proposed and at the same time the adequate instruction for the rural population to start a rapid treatment of the accidents by venomous snakes.

III

IMUNOLOGIA

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24. PHARMACOLOGICAL EFFECTS OF THE VENOM OF *HAPALOCHLAENA MACULOSA*

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The octopus *Hapalochlaena maculosa*, banded octopus, was pinned out on cork and the brain pierced with a sharp instrument. The venom gland on each side was dissected. The gland was weighed and ground up with silica in Tyrode. Initial studies have been carried out with this Tyrode extract and more specialized chromatography is being done on an acid acetone extract.

Fig. 1 — Dissected venom glands removed from the Octopus.

In Australia fatal cases of poisoning from handling this octopus have occured and some near fatal cases. The beak like process allows venom injection. The most obvious physical feature is respiratory paralysis and prolonged artificial respiration can be expected to end in survival.

The venom gland is the posterior salivary gland and the banding on the octopus is not unlike that on the cone shell and the tiger snake which also produce neurotoxin.

The isolated jejunum of the guinea pig was tested for activity and the responses are similar to those produced by snake venom. They differ in that this venom greatly reduces the sensivity of the gut to histamine and contraction to the venom is immediate. Following the immediate contraction there is a typical delayed S.R.S. reaction such as we have in anaphylaxis and with snake venom. We are now engaged in purification studies to dissociate these responses.

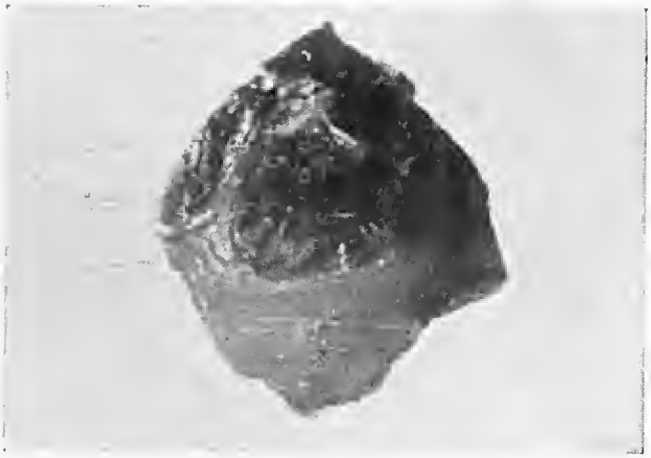

Fig. 2 — Beak like process attached to the terminal ducts of the gland.

Fig. 2a — View of beak like process indicating the solidity of the beak allowing skin penetration.

Fig. 3 — View of Octopus showing banding on tentacle.

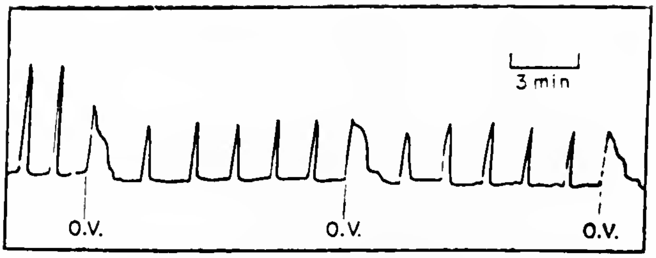

Fig. 4 — Responses of isolated jejunum of guinea-pig O.V. 4.5 mg Venom extract showing immediate contraction followed by delayed relaxation. Unlettered contractions to 0.1 μg histamine. Halving of responses after venom injection.

When we perfuse the isolated heart of the cat we find that venom in doses quite effective on the gut produce very little effect on the heart. The amplitude of contraction falls off only slightly more rapidly than that of control hearts. The coronary flow is also little affected even when the venom is recirculated with the perfusion. The rate of the heart is slowed immediately following injection of venom but this recovers rapidly. The ECG under the Langendorf type perfusion with Tyrode normally soon shows block in contra distinction to that of the blood perfused heart which is normal. We were not able therefore to determine with this technique whether heart block could occur from a direct effect of the venom and its appearance in the intact animal follows probably from anoxia.

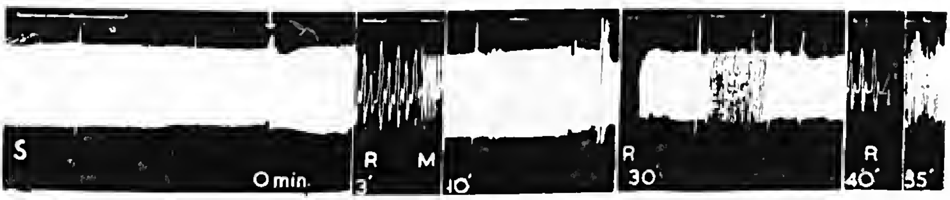

Fig. 5 — Contraction of the isolated perfused heart of the cat. At arrow, intracoronary injection of venom (18 mg). There is little fall off of amplitude over 40 minutes. Time S = 1 min, R = 1 sec, M = 10 sec intervals.

In the intact cat following the injection of gland extract intravenously the respiration slows and the blood pressure declines slightly. Sometimes respiration stops, returns temporarily, and finally ceases a few minutes later. Large doses of gland extract lower the blood pressure significantly and this is coincidental with failure of respiration. Artificial respiration and cardiac massage have not been adequate to restore the animal in acute experiments.

The rat diaphragm preparation is very sensitive to venom extract. The response to indirect stimulation is rapidly abolished. At a later stage the response to indirect stimulation fails and recovery on repeatedly washing out the bath occurs in reverse order; but the response to nerve stimulation remains impaired for a very long time.

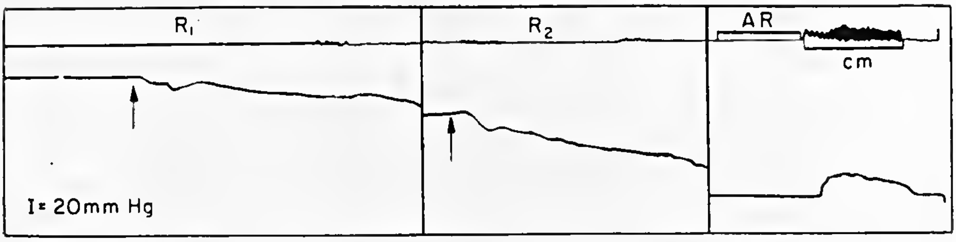

Fig. 6 — Upper tracing — respiration. Lower tracing — blood pressure, in the intact cat. At the arrows, venom extract injected, 18,50 mg gland extract. Respiration after temporary increase soon fails. Blood pressure slowly declines with temporary depression from extract. Respiration fails completely before heart beat fails.

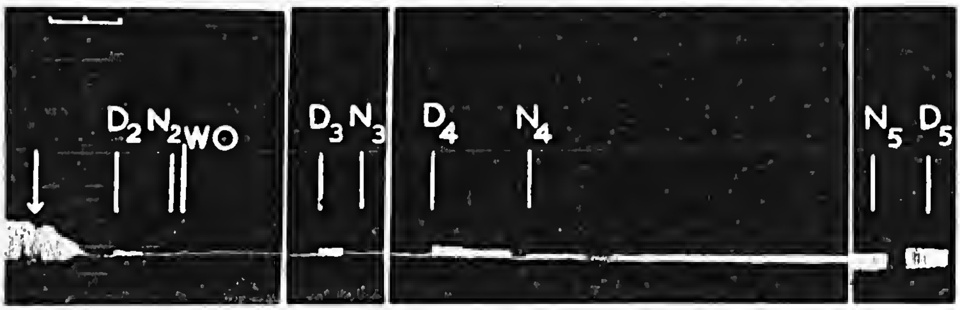

Fig. 7 — Responses of rat diaphragm to (D) direct and (N) indirect (phrenic nerve) stimulation. Failure to direct stimulation is not complete nor as severe as that to indirect stimulation, and recovers much more rapidly.

It is apparent that the main effect of the venom is respiratory paralysis. Cardiac effects are largely secondary, probably due to anoxia, and appear in the intact animal but to no marked degree in the isolated heart. Respiration fails before the blood pressure falls significantly. One would expect the blood pressure to rise with the failure of respiration from asphyxia unless the venom has a similar effect on the vaso-motor centre or peripheral arteriolar inervation as it has on the rat diaphragm. It may well be that there is a central effect on respiration also and we shall investigate this possibility later. The temporary slowing of the heart but without impaired amplitude of beat suggests some direct cardiac effect similar to that of the venom on the muscle of the diaphragm and the gut.

The isolated diaphragm progressively shows failure of transmission of the nerve impulse as well as less evident direct muscle depression and since this effect is rapid and marked in relation to quantity of gland compared with any cardiac effect it would appear that the main effect of the venom is in blocking nerve conduction and so causing death. It would therefore appear advisable in man in addition to applying artificial respiration to administer prostigmin, and thereby enhancing transmission.

Chromatographic studies indicate there are two muscular toxins.

25. DIFERENCIAS INMUNOLÓGICAS ENTRE ENTIDADES SIMPÁTRIDAS DE ARAÑAS DEL GÉNERO *LATRODECTUS* WALCKENAER

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La sistemática de las arañas del género *Latrodectus* resulta sumamente compleja, especialmente en relación al grupo *mactans*.

En la actualidad parece haberse llegado a un punto en el que la utilización exclusiva de los caracteres somáticos no aportaría más elementos de juicio para dilucidar este problema.

Así, mientras ciertos aracnólogos como Schiapelli y Pikelin(1, 2) sostienen que en la Argentina solamente existe una especie, *L. mactans* (Fabricius), 1775, tal como fuera establecido en la tesis de Sampayo de 1942 (3) y en trabajos posteriores, otros, por el contrario, como Levi (4) y luego Abalos (5) opinan que en nuestro país se encuentran varias especies afines (gemelas o crípticas) a *L. mactans*. Este ultimo autor utiliza la designación provisoria de *Latrodectus N.º 1, N.º 2, N.º 3* y *N.º 4* para las 4 formas del grupo *mactans*, según él de jerarquía específica, encontradas en la provincia de Santiago del Estero. Fundamenta su diferenciación en su ecología y principalmente en las características constantes de las ooteeas.

Dadas las diferentes opiniones y ante la posibilidad de obtener apreciables cantidades de ejemplares de estas arañas, resolvimos encarar el problema desde el punto de vista inmunológico, con el fin de obtener nueva información en torno a esta controversia. En este sentido y con similares propósitos merece citarse la reciente contribución de Mc Crone y Netzloff (6) sobre las propiedades inmunológicas y electroforéticas de los venenos de las especies norteamericanas del género *Latrodectus*.

Material y métodos

Se investigaron las precipitinas por el método de la doble difusión en agar, según Outcherlony (7) y Grasset y colab. (8) entre otros, por su sencillez, posibilidades de trabajar con pequeñas cantidades de material y por la reproductibilidade de sus resultados.

Se emplearon como antígeno extractos acuosos de aparatos venenosos de ejemplares hembras (100 aparatos por ml de solución salina) de tres entidades que conviven en la ciudad de Santiago del Estero y aledaños, a saber: *Latrodectus geometricus*, especie perfectamente identificable y sobre cuya clasificación no existe problema alguno; *Latrodectus N.º 1 y Latrodectus N.º 2* (de Abalos), entidades sobre cuyo "status" existem discrepancias (debido a su menor abundancia en esa zona no hemos podido investigar, por ahora, *Latrodectus N.º 3* y

Latrodectus N.º 4). Por los motivos que se expondrán más adelante, y a los efectos de fundamentar nuestras conclusiones, consideramos de capital importancia que todos los ejemplares de *Latrodectus N.º 1* y *Latrodectus N.º 2* procedieran de la misma área, vale decir que fueran simpátridas.

Como anticuerpos se utilizaron los obtenidos en suero de caballo inmunizado con cantidades no determinadas de aparatos venenosos de *Latrodectus N.º 1* y *N.º 2*, puesto que durante el proceso de inmunización no se tuvo en cuenta la posibilidad de que se tratara de dos entidades diferentes. El título del suero es tal que 1 ml protege contra 150 DMM ratón (1 DMM = 0.005 ml de una solución que contiene 32 ap. ven./ml) por vía endovenosa, posteriormente éste fue diluído al doble en solución fisiológica para su utilización en estas experiencias.

Resultados

Los espectros de las líneas de precipitinas de la figura 1 evidencian que si bien las tres entidades poseen ciertos antígenos idénticos, existen otros no comunes a *L. geometricus* por un lado y a *L. N.º 1* y *L. N.º 2* por otro; esto es bien notorio por los entrecruzamientos observables en las zonas de contacto.

Igualmente se verifican diferencias antigénicas, aunque de grado menor, entre *Latrodectus N.º 1* y *N.º 2*. En la figura 2 se observan las líneas principales A, B, C y D, en general desdobladas, que corresponden a los antígenos similares comunes y la línea B' característica de *Latrodectus N.º 2* y que no existe en *Latrodectus N.º 1*, como puede apreciarse en los tres lugares señalados.

Además debemos hacer notar que existen otras líneas de menor intensidad que aparecen más próximas a la cúpula del suero que tampoco corresponden a antígenos idénticos de ambos extractos.

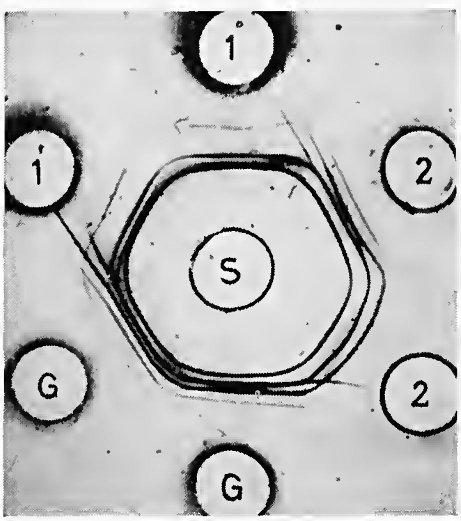

Fig. 1 — Espectros de bandas de precipitinas de aparatos venenosos de *Latrodectus*. 1: (*mactans*) N.º 1; 2: (*mactans*) N.º 2; G: *geometricus*; S: suero antilatrodectus.

Finalmente señalaremos que, en lo que se refiere a la formación del espectro existen significativas diferencias en el orden cronológico y en la intensidad con que aparecen las líneas en ambos espectros. Este hecho habla a favor de ciertas diferencias cuantitativas y de velocidad de difusión existentes aún en los antígenos comparables y considerados como similares de ambos extractos.

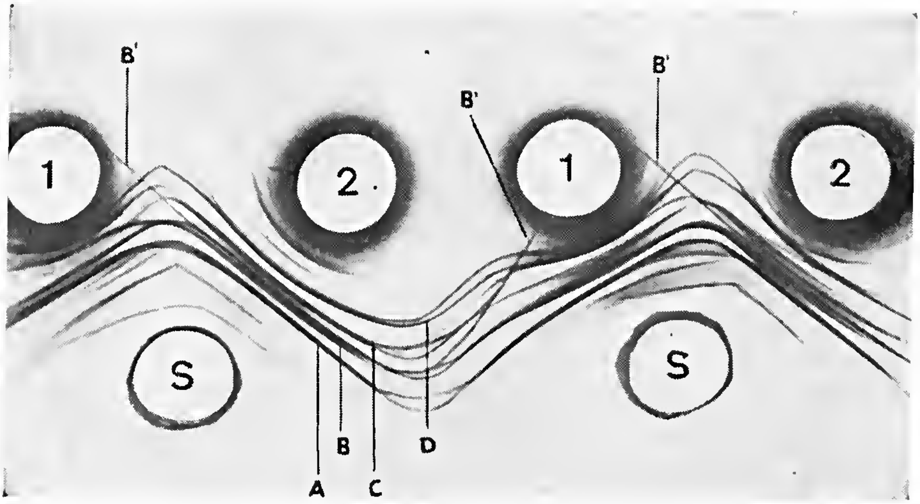

Fig. 2 — Espectros de bandas de precipitinas de aparatos venenosos de *Latrodectus*. 1, 2 y S igual a Fig. 1. A, B. C y D: bandas correspondientes a antígenos comunes. B': banda correspondiente a un antígeno no común.

Comentário

De lo anteriormente expuesto surge la no total identidad antigénica entre las entidades *Latrodectus N.º 1* y *N.º 2*, sobre las que tratamos de aportar nuevos elementos de juicio relativos al esclarecimento de su "status".

Ahora bien, de acuerdo a las investigaciones con venenos ofídicos de Schenberg (9, 10) y nuestras (11, 12), se ha podido comprobar que diferencias antigénicas de la magnitud señalada son registrables *dentro de una misma especie* al comparar muestras procedentes de diferentes poblaciones separadas geográficamente, vale decir alopátridas.

Teniendo en cuenta estos antecedentes es que tuvimos especial cuidado en tomar, para nuestras investigaciones, material de individuos de *Latrodectus N.º 1 y N.º 2* de la misma procedencia, vale decir que fueran simpátridas, o más exactamente microparapátridas, según la reciente denominación propuesta por Hobart M. Smith (13). A este respecto es objetable que en el trabajo de McCrone (op. cit.), no se haga mención con exactitud a la procedencia del material estudiado, puesto que no tendrían validez específica "per se" las diferencias inmunológicas halladas en ejemplares alopátridos. Como en este tipo de relación espacial el intercambio genético es posible, de no producirse éste, debe admitirse forzosamente la existencia de un aislamiento en su reproducción. En efecto, esto es lo que pensamos que acontece por haber comprobado que ambas entidades mantienen constantemente diferenciados sus componentes antigénicos a pesar de la proximidad de sus nichos ecológicos.

En síntesis, somos de opinión que, a las distintas preferencias ecológicas y de comportamiento mencionadas por Abalos (op. cit.) deben sumarse las que ahora señalamos en relación a la diferente composición antigénica de sus respectivos aparatos venenosos. Estos hechos deben ser indudablemente referidos a la constitución desigual del genoma respectivo y son prueba de la existencia de mecanismos de aislamiento pre y/o postnupciales que permiten suponer que

estas dos entidades en su diferenciación ya han alcanzado un rango específico. Cuando se prosigan las investigaciones en otros lugares se estará en condiciones de apreciar cual es la amplitud de la dispersión geográfica de cada una de estas dos formas y cuan extensa es su área de simpatría.

Summary

By means of double-diffusion-tests the homogenates of the venom from female *Latrodectus* spiders, belonging to the species *geometricus* Koch and *L. N.º 1* and *N.º 2* (*mactans* group of Abalos) whose status is questioned, were studied. These spiders came from Santiago del Estero city and surroundings. The precipitin spectrum shows that the three entities possess several common and certain uncommon antigenic components. The immunochemical differences between the two sympatric (or microparapatric) entities *Latrodectus N.º 1* and *Latrodectus N.º* 2 and their ecological and ethological characteristics prove the existence of isolating mechanism. These facts allow us to conclude that these entities could have to be raised to specific level.

Agradecimiento — Quedamos muy reconocidos al Dr. Jorge W. Abalos por la provisión de numerosos ejemplares vivos de las diferentes entidades de *Latrodectus* empleadas en este trabajo, así como de sus aparatos venenosos ya desecados.

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26. ESTUDIO COMPARATIVO MORFOLÓGICO E INMUNOLÓGICO ENTRE LAS DIFERENTES ENTIDADES DEL GÉNERO *MICRURUS* WAGLER (*OPHIDIA, ELAPIDAE*) DE LA ARGENTINA

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En relación a las corales del género *Micrurus* diversos autores han intentado elucidar el problema sistemático de las formas del sur del continente, a saber: Schmidt (1), Amaral (2), Vanzolini (3), Shreve (4) y Pradèl Azevedo (5).

Es obvia la conveniencia de que el enfoque de dicho tema sea hecho en forma integral tratando comparativamente todas las entidades del continente con el objeto de poder apreciar debidamente su dispersión geográfica, sus áreas de contacto, de simpatría y de intergradación; sus afinidades morfológicas, probables relaciones filogenéticas, etc.

Este criterio, el más acertado en relación a este grupo, es seguido con promisorios resultados, según nuestra información, por Janis Roze, actualmente en Washington. No obstante, pensamos que, a veces, ciertos aspectos parciales del problema, restringidos a zonas determinadas pueden tratarse independientemente con resultados valederos y con posibilidades de ser luego incorporados al considerarse el género en su totalidad. Es por este motivo que hemos encarado el asunto encuadrándolo dentro de los límites de nuestro país, de acuerdo a las facilidades de obtención de material fidedigno y en series apreciables como para permitir el tratamiento estadístico de su morfología. Pero, la razón fundamental que nos restringió a la consideración exclusiva de las entidades que habitan en la Argentina fue nuestra intención de extender comparativamente su estudio al análisis inmunológico de sus respectivos venenos. Para ello era menester contar con muestras de ponzoña originarias de ejemplares ciertamente individualizados, tanto desde el punto de vista de sus caracteres ectosomáticos, como de su procedencia. Además, con el objeto de que nuestros datos puedan ser ulteriormente confrontados con los proporcionados por otras muestras de venenos, siguiendo las mismas técnicas, hemos empleado en el sistema antígeno-anticuerpo un suero ya standardizado, de uso terapéutico y por consiguiente de fácil obtención.

Existen en la Argentina cuatro entidades del género *Micrurus*. El área mayor corresponde a la forma que preferimos llamar, por los motivos que más adelante se expondrán, *Micrurus frontalis pyrrhocryptus* (Cope), que ocupa la totalidad de las provincias situadas al oeste de los ríos Paraguay y Paraná y al norte del paralelo 36. En las provincias de Entre Ríos y Corrientes situadas al este del río Paraná y al oeste del río Uruguay se distribuye una nueva subespecie que hemos denominado *Micrurus frontalis mesopotamicus*. Finalmente, en la proeincia de Misiones, situada en el confín noreste del país y también entre

los ríos precitados, conviven *Micrurus frontalis altirostris* (Cope) y *Micrurus corallinus* (Wied). Por lo que antecede, se pone bien en evidencia que los ríos Paraguay y Paraná y en parte el Uruguay constituyen efectivos lindes que delimitan netamente cada una de las formas, debiéndose a esta causa la inexistencia de intergradaciones, las que justamente se producen en las zonas de contacto entre *M. f. altirostris* y *M. f. mesopotamicus* donde non existen dichas barreras (Fig. 8).

Material y métodos

El material básico de nuestro estudio lo constituyeron ejemplares del género *Micrurus* que llegaron vivos al Instituto Nacional de Microbiología "Carlos G. Malbrán", procedentes de distintas localidades del país. Estos fueron sometidos a extracciones periódicas de veneno; luego de un lapso de unos cuatro meses fueron sacrificados y fijados, e ingresaron en nuestras colecciones (CHINM). Además se examinó el material existente en las colecciones herpetológicas del Museo Argentino de Ciencias Naturales "Bernardino Rivadavia" de Buenos Aires (MACN), del Instituto Butantan de São Paulo, del Museu Riograndense de Ciencias Naturais de Pôrto Alegre (MRCN), y del Museo de Historia Natural de Montevideo (MHNM) a cuyos respectivos jefes, Jorge Cranwell, Alphonse R. Hoge, Thales de Lema y B. Orejas Miranda quedamos muy reconocidos.

Se investigaron las precipitinas por el método de la doble difusión en agar según Outcherlony (6), Grasset y col. (7) y otros, y microdifusión utilizado por Schenberg (8, 9).

Las muestras de veneno extraído de cada ejemplar fueron desecadas y conservadas por separado. Se utilizó una solución que contenía 5 mg de veneno en 0.75 ml de solución fisiológica.

Se empleó el suero antielapídico elaborado por el Instituto Butantan contra veneno de *Micrurus frontalis* y *Micrurus corallinus*, con un título tal que 1 ml neutraliza 3 D.L.M. de veneno elapídico; en este caso fue diluído al 50% en solución fisiológica.

Resultados

Análisis inmunológico

El método de la doble difusión en agar nos permitió poner en evidencia que existen entre los venenos de las cuatro entidades de *Micrurus* consideradas, algunos antígenos no comunes, mientras que se aprecian otros que exiben una total o parcial identidad entre sí. Según puede observarse en las Figs. 1, 2 y 3 hay un cierto número de bandas de precipitinas comunes a las tres subespecies de *Micrurus frontalis* (*pyrrhocryptus, mesopotamicus* y *altirostris*), a la par que tal identidad no parece ser tan absoluta entre dichas subespecies y *M. corallinus*. También es dable observar la presencia de anchas bandas internas (véase Figs. 1, 2 y 3) peculiares y exclusivas de cada una de las entidades, tanto en lo que respecta a cada subespecie de *Micrurus frontalis* como en *M. corallinus*.

Esta última comprobación no debe extrañar, puesto que ya Schenberg (8, 9) halló variaciones de esta naturáleza, aunque no de esta magnitud entre diferentes subespecies de *Bothrops neuwiedi* e inclusive entre poblaciones diferentes de *Bothrops jararaca*.

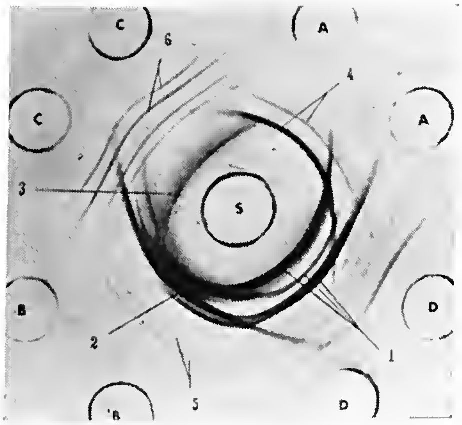

Fig. 1 — Bandas de precipitinas. A: *M. corallinus* (proc. Puerto Esperanza, Misiones), B: *M. f. pyrrhocryptus* (proc. La Posta, Córdoba), C: *M. f. mesopotamicus* (proc. El Cimarrón, Entre Rios), D: *M. f. altirostris* (proe. Puerto Mineral, Misiones), S: suero antielapidico Inst. Butantan. 1: bandas comunes a los venenos D y B, 2: banda exclusiva veneno B, 3: banda exclusiva veneno C, 4: bandas exclusivas veneno A. 5 y 6: bandas comunes a B, C y D.

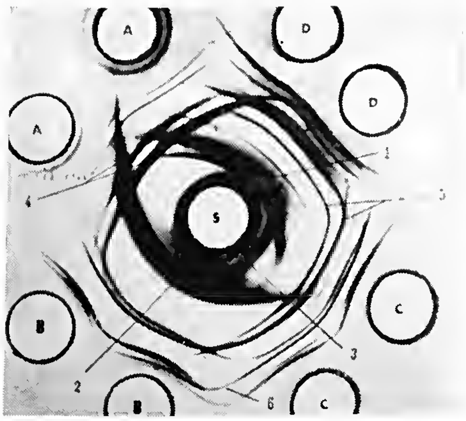

Fig. 2 — Bandas de precipitinas. A: *M. corallinus* (proe. Puerto Esperanza, Misiones), B: *M. f. pyrrhocryptus* (proc. Averias, Santiago del Estero), C: *M. f. mesopotamicus* (proc. El Cimarrón, Entre Rios), D: *M. f. altirostris* (proc. Puerto Mineral, Misiones), S: suero antielapidico Inst. Butantan. 1: banda exclusiva del veneno D, 2: banda exelusiva del veneno B, 3: banda exelusiva del veneno C, 4: bandas exelusivas del veneno A, 5 y 6: bandas comunes a B, C y D.

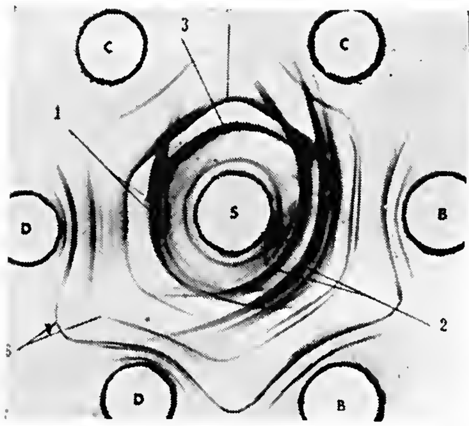

Fig. 3 — Bandas de precipitinas. B: *M. f. pyrrhocryptus* (proc. Averias, Santiago del Estero), C: *M. f. mesopotamicus* (proc. El Cimarrón, Entre Ríos), D: *M. f. altirostris* (proe. Oberá, Misiones), S: suero antielapidico Inst. Butantan. 1: bandas exelusivas del veneno D, 2: bandas exclusivas del veneno B, 3: banda exclusiva del veneno C, 5: bandas comunes a los venenos C, D y B, 6: bandas comunes a los venenos D, B y C aunque poco visible en este último.

En resumen, nos parece que la divergencia inmunológica de los venenos de las entidades de *M. frontalis* entre sí. es de menor grado que la comprobada entre ellas y *M. corallinus.* Estos datos, como se verá por lo que sigue, son concordantes con los datos morfológicos estudiados.

Debemos hacer notar que se comprobó una significativa homogeneidad inmunológica entre las diversas muestras de venenos pertenecientes a una misma entidad, a pesar de que la existencia de algunas débiles líneas de precipitinas no fue constante en todas las muestras procedentes de distintas localidades.

Análisis morfológico

Micrurus frontalis pyrrhocryptus (Cope) 1862

Denominamos así a esta entidad de acuerdo al criterio sustentado por Shreve (4), que concuerda perfectamente con nuestras propias conclusiones, tanto desde el punto de vista de la morfología como de la composición de su veneno. Vale decir que, en primer lugar, no compartimos la posición de Amaral (2) en el sentido de que esta entidad deba considerarse una subespecie de *M. lemniscatus* y, en segundo lugar pensamos que, aunque con características propias bastante peculiares, no ha alcanzado aún el nivel suficiente como para separarla de *frontalis*, máxime que hasta el momento no hemos podido comprobar la simpatría de ambas formas. Pero, lo que nos condujo a definirnos más categóricamente en este sentido, fue el hallazgo de la subespecie *M. f. mesopotamicus* que constituye, como se verá, una raza con caracteres intermedios entre *M. f. pyrrhocryptus* y *M. f. altirostris*.

Como se aprecia en los gráficos adjuntos (Figs. 4 y 5) *M. f. pyrrhocryptus* posee los siguientes caracteres en sua escutelación: dorsales 15; ventrales, ♂♂ 214-240, ♀♀ 218-238; subcaudales, ♂♂ 25-30, ♀♀ 21-29; nucales 4-11; número de triadas del cuerpo 5-11.

Distribución geográfica — Comprende las siguientes provincias argentinas: Formosa, Chaco, Santa Fe, Córdoba, San Luis, Mendoza, La Pampa, San Juan, La Rioja, Catamarca, Tucumán, Santiago del Estero, Salta y Jujuy, extendiéndose al Chaco paraguayo, sudeste de Bolivia y sur de Mato Grosso (Brasil).

Material estudiado — CHINM: N.° 703, Estanislao del Campo, Formosa, 18-XII-53; N.° 704, La Verde, Chaco, 29-I-54; N.° 706, Cañada de Luque, Córdoba, 22-IV-57; N.° 708, Chalacea, Córdoba; N.° 725, Quitilipí, Chaco, 29-II-56; N.° 1354, Los Juríes, Santiago del Estero, 29-III-61; N.° 1472, Resistencia, Chaco, 29-VI-62; N.° 1479, Naré, Santa Fe, 8-VIII-62; N.° 1480, Las Avispas, Santa Fe, 29-X-62; N.° 1513, Río Piedras, Salta, 22-III-63; N.° 1553, Pirané, Formosa, 20-V-63; N.° 1660, Padre Lozano, Salta, 23-IV-64; N.° 1667, La Banda, Santiago del Estero, 18-V-64; N.° 1713, Atahona, Tucumán, 9-X-64; N.° 1736, Haase, Santiago del Estero, 18-V-64; N.° 1738, Lucila, Santa Fe, 14-X-64; N.° 1782, Padre Lozano, Salta, 9-X-64; N.° 1786, Lucila, Santa Fe, 2-XII-64; N.° 1840, Formosa, Formosa, 19-VII-65; N.° 1845, Embarcación, Salta, 2-VIII-65; N.° 1853, Chorotis, Chaco, 24-V-65; N.° 1866, Formosa, Formosa, 13-VIII-65; N.° 1878, La Posta, Córdoba, 14-V-65; N.° 1907, Chilecito, La Rioja, 1-II-65; N.° 1982, Siján, Catamarca, 26-IV-66; N.° 1997, Formosa, Formosa, 20-IV-66.

MACN: N.° 4699, Sur de la Sierra, San Luis, 8-VII-1925; N.° 5147-5148-5149, Catamarca, 16-III-1928; N.° 6897, La Rioja, 23-X-1934; N.° 7738, La Paz, Mendoza, 22-X-1941; N.° 8863, Santa Rosa, La Pampa, 22-XII-46.

Micrurus frontalis mesopotamicus n. ssp.

Por su dispersión geográfica neta, así como por los caracteres morfológicos e inmunológicos de su veneno, constituye una entidade bien definida. Como se dijo precedentemente exhibe rasgos intermedios entre la forma con la que colinda hacia el oeste (*M. f. pyrrhocryptus*) y con la que contacta al norte y al este (*M. f. altirostris*).

Descripción del holotipo — CHINM N.º 1823, macho adulto. Longitud total 82,6 cm, Villa Federal, Entre Ríos, Argentina, 28-IV-65; J. Vermeersch leg.

Coloración: Cabeza negra, una ancha franja blanquecina transversal que comprende las escamas prefrontales, loreales y mitad posterior de las nasales; una angosta línea blanca que bordea la parte anterior de las parietales y se continúa hasta las labiales superiores; parietales negras con el extremo posterior rojo. Labiales y región gular blanquecina con pequeñas manchas oscuras irregularmente distribuídas.

Escamas dorsales 15; ventrales 218; subcaudales 24; nucales 3; número de triadas del cuerpo 13; el anillo negro central es aproximadamente dos veces más ancho que los laterales y casi igual al espacio rojo intermedio.

Alotipo CHINM N.º 1512, hembra adulta. Longitud total 73,5 cm. El Cimarrón, Entre Ríos, Argentina, 26-IV-63.

Las diferencias de coloración con el holotipo son las siguientes: escamas parietales totalmente negras, la línea blanquecina característica del borde anterior de los parietales se presenta discontínua. Escamas ventrales 225; subcaudales 22, nucales 3, número de triadas del cuerpo 12.

Paratipos — ♂♂ CHINM N.º 1783, 1861, 1871, 1952, 1983, 2009; ♀♀ CHINM N.º 1525, 1627, 1720, 1994, 2006, 2008. Semejantes al holo y alotipo presentan variaciones en la nitidez de la línea branquecina del borde anterior de los parietales, número de ventrales, subcaudales, nucales, número de triadas del cuerpo y la relación entre el ancho de sus anillos negros centrales y laterales, con valores que oscilan dentro de los límites señalados en los gráficos correspondientes (véase Figs. N.º 4 y 5).

En un principio pensamos que la relación entre el ancho del anillo negro central y los laterales era menor en esta subespecie que en *M. f. pyrrhocryptus*, pero el análisis estadístico demostró que dicha diferencia no es significativa.

Distribución geográfica — Provincias de Entre Ríos, Corrientes y sudoeste de Misiones (Argentina). En las zonas limítrofes (NE de Corrientes y SO de Misiones) aparecen esporádicamente intergradaciones.

Material estudiado — CHINM: N.º 740, Saladas, Corrientes; N.º 743, San Ignacio, Misiones, 26-IV-48; N.º 1512, El Cimarrón, Entre Ríos, 26-IV-63; N.º 1521, Villa Federal, E. Ríos, 31-I-62; N.º 1522, Paraná, E. Ríos, 31-I-62; N.º 1523, El Cimarrón, E. Ríos, 11-I-62; N.º 1524, Santa Elena, E. Ríos, 17-IV-62; N.º 1525, El Cimarrón, E. Ríos, 2-IV-62; N.º 1526, El Cimarrón, E. Ríos, 31-I-62; N.º 1627, Chajarí, E. Ríos, 20-V-64; N.º 1720, Santa Lucía, Corrientes, 15-IV-64; N.º 1783, El Cimarrón, E. Ríos, 17-XII-64; N.º 1823, Villa Federal, E. Ríos, 28-IV-65; N.º 1861, San Roque, Corrientes, 24-V-65; N.º 1871, Villa Bovril, E. Ríos, 30-III-65; N.º 1952-1967-1969, El Cimarrón, E. Ríos, 9-III-66; N.º 1983, El Cimarrón, Entre Ríos, 25-II-66; N.º 1994, El Cimarrón, E. Ríos, 27-I-66; N.º 2003, San Roque, Corrientes, 2-II-62; N.º 2004, San Roque, Corrientes, 12-I-62; N.º 2005, Santo Tomé, Corrientes, 30-X-61; N.º 2006, El Cimarrón, E. Ríos, 9-III-65; N.º 2007, El Cimarrón, E. Ríos, 15-II-66; N.º 2008, Santa Lucía, Corrientes, 4-V-66; N.º 2009, El Cimarrón, E. Ríos, 11-IV-66. MACN: N.º 6427, Corrientes, 1923; N.º 6575, Santa Elena, E. Ríos, 1933; N.º 8629, Paraná, E. Ríos, 1946.

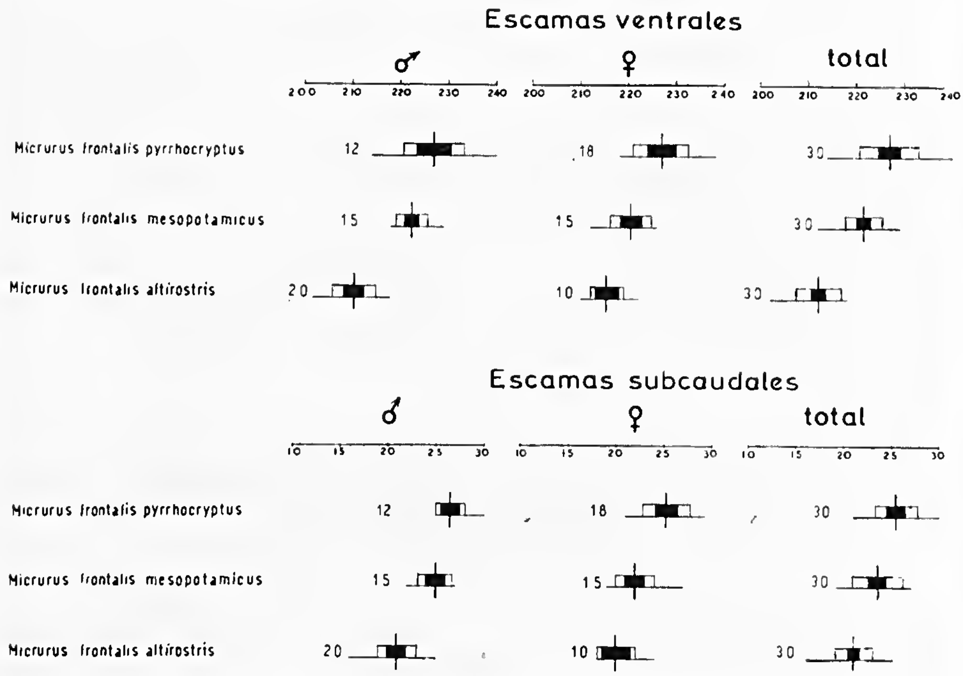

Fig. 4 — Análisis estadístico. La línea horizontal corresponde al rango, la vertical a la media, el rectángulo negro a dos errores standard y el rectángulo blanco a una desviación standard.

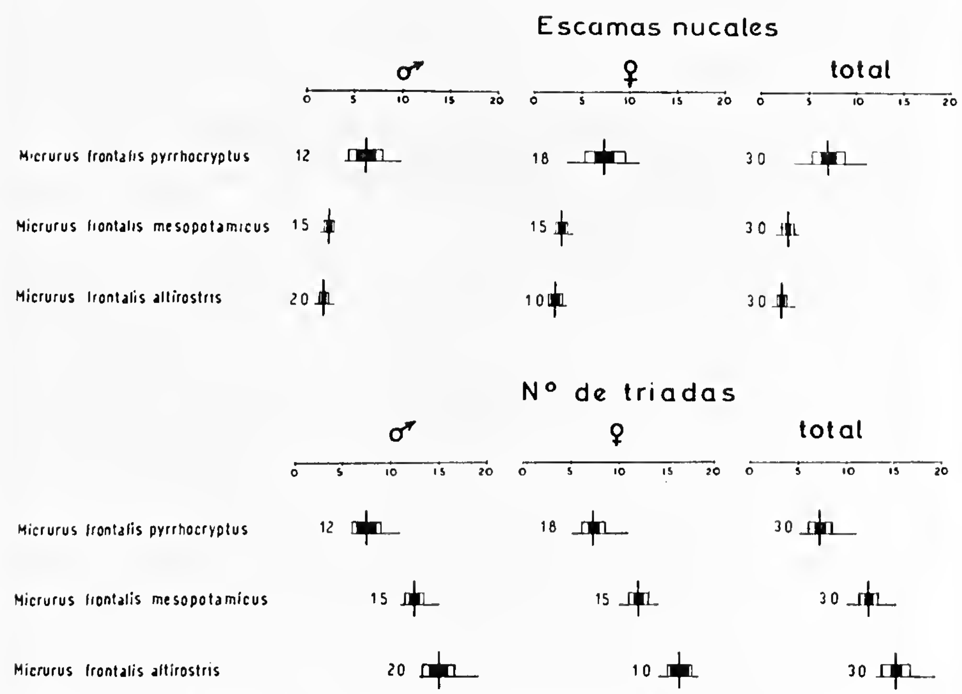

Fig. 5 — Análisis estadístico. Referencias igual a Fig. 4.

Micrurus frontalis altirostris (Cope) 1859

La determinación de esta forma ofreció algunas dificultades puesto que entorpeció nuestra tarea la existencia de una subespecie posterior de Amaral (*M. f. multicinctus*) 1944, que presenta evidentemente muchas afinidades y áreas de superposición. El examen del tipo, paratipo y otros ejemplares, realizado en el Instituto Butantan, así como material de la colección del Museu Riograndense de Ciências Naturais, nos decidió a no aceptar la validez de esta forma. Compartimos el criterio adoptado por Shreve (4) en el sentido de que esta entidad exhibe rasgos de *f. altirostris* y *f. frontalis*. En efecto, muchos ejemplares que observamos son intergradaciones entre las precitadas subespecies, de allí que el material proveniente de localidades más meridionales se acerque a *f. altirostris* y el de más al norte a *f. frontalis*. Todo ello hace imprecisa su caracterización y definición. El estudio comparativo con el material procedente del Museo de Historia Natural de Montevideo, Uruguay, nos permitió sin lugar a dudas clasificar nuestra forma como perteneciente a *M. f. altirostris*, dada la total coincidencia en la escutelación y en los patrones de diseño, aunque debemos consignar que los ejemplares misioneros son algo menos melánicos que los del Uruguay.

Sus vinculaciones con *M. f. frontalis* son evidentes, pero puede ser separada de ésta por el número de escamas ventrales que varían entre: ♂♂ 216-236, ♀♀ 221-242 en *M. f. frontalis* y entre: ♂♂ 201-219, ♀♀ 210-221 en *M. f. altirostris* y las triadas del cuerpo que oscilan entre 9 y 15 en la primera y entre 13-19 en la segunda (véase Figs. 4 y 5).

En cuanto a su colorido debemos mencionar que contrariamente a lo que sucede en *M. f. frontalis*, donde las parietales son negras y sólo por excepción

Fig. 6 — *Micrurus frontalis mesopotamicus*. Paratipo n.º 1627.

su margen anterior es de color claro, en *M. f. altirostris* presentan en forma constante una ancha zona anterior clara.

Los restantes caracteres de escutelación de *M. f. altirostris* son los siguientes: subcaudales, ♂♂ 16-25, ♀♀ 18-24, nucales 1-3.

Distribución geográfica — Provincia de Misiones (Argentina), Uruguay, Paraguay y los Estados brasileños de Rio Grande do Sul y Paraná.

Material estudiado — *Argentina*. CHINM: N.° 727, Puerto Esperanza, Misiones, 21-I-49; N.° 1361, Eldorado, Misiones, 26-IX-60; N.° 1605, Posadas, Misiones, 30-IX-63; N.° 1670, Eldorado, Misiones 7-VII-64; N.° 1689, Posadas, Misiones, 19-X-64; N.° 1802, Puerto Paranaí, Misiones, 23-II-65; N.° 1847, Puerto Mineral, Misiones, 13-X-65; N.° 1920, Puerto Mineral, Misiones, 13-X-65; N.° 1928, Puerto Azara, Misiones, 29-X-65; N.° 1940, Oberá, Misiones, 8-X-62; N.° 1941, Oberá, Misiones, 29-XI-65; N.° 1942-1943, Oberá, Misiones, 10-XII-65; N.° 1946, Oberá, Misiones, 3-I-66; N.° 1953, Oberá, Misiones, 15-IV-66; N.° 1980, Leandro N. Alem, Misiones, 3-I-66; N.° 1981, Oberá, Misiones, 5-IV-66; N.° 1984, L. N. Alem, Misiones, 26-IV-66; N.° 1992, Oberá, Misiones, 18-I-60; N.° 1999, Posadas, Misiones, 18-XII-61; N.° 2000, Puerto Esperanza, Misiones, 26-IX-61; N.° 2001, Puerto Esperanza, Misiones, 11-X-61; N.° 2002, Eldorado, Misiones 3-IV-62. MACN: N.° 14a (2 ej.), San Ignacio, Misiones, 1918; N.° 1602, Misiones, 1902; N.° 2447, San Ignacio, Misiones, 22-XII-1913; N.° 7785, San Ignacio, Misiones, 20-IV-1942; N.° 9486, Puerto Londera, Misiones, IX-1947; N.° 12647, Puerto Bemberg, Misiones, II-1950.

Uruguay. MHNM: N.° 0977, San Gregorio, Puntas Arroyo Chamizo, San José, 29-I-1965; N.° 0979, Tambores, Tacuarembó, II-66; N.° 1116, Catalán Chico, Artigas, 13-III-65.

Material estudiado de *Micrurus frontalis multicinctus* (Amaral): Inst. Butantan, Tipo N.° 8877, Teixeira Soares, Paraná, Brasil; Paratipo N.° 7874, Iratí, Paraná, Brasil; MRCN N.° 1789, Viamão, Rio Grande do Sul, Brasil; N.° 2656, prob. Viamão, Brasil; N.° 2658, Estrada de Taquara, Brasil.

Micrurus corallinus (Wied) 1820

Es ésta la más pequeña de las formas que habitan nuestro país. No se halla estrechamente relacionada con las precedentes, tal se desprende del estudio de su morfología y de las propiedades inmunológicas de su veneno. Sus anillos negros simples y bordeados de blanco amarillento se presentan en un número que oscila entre 16 y 20. Escamas dorsales en 15 filas, el número de escamas ventrales y subcaudales varía notablemente según el sexo: ventrales ♂♂ 203-205, ♀♀ 216-220; subcaudales ♂♂ 44-46, ♀♀ 29-31.

Distribución geográfica — Provincia de Misiones (Argentina), Paraguay, sur del Brasil.

Material estudiado — CHINM N.° 697-698, Puerto Esperanza, Misiones, 21-I-49; N.° 699, Puerto Esperanza, Misiones, 6-X-49; N.° 700, Puerto Tabay, Misiones, 19-X-55; N.° 1273, Posadas, Misiones, 20-X-61; N.° 1432, Puerto Esperanza, Misiones, 30-I-62; N.° 1530, Posadas, Misiones, 29-VII-63; N.° 1911,

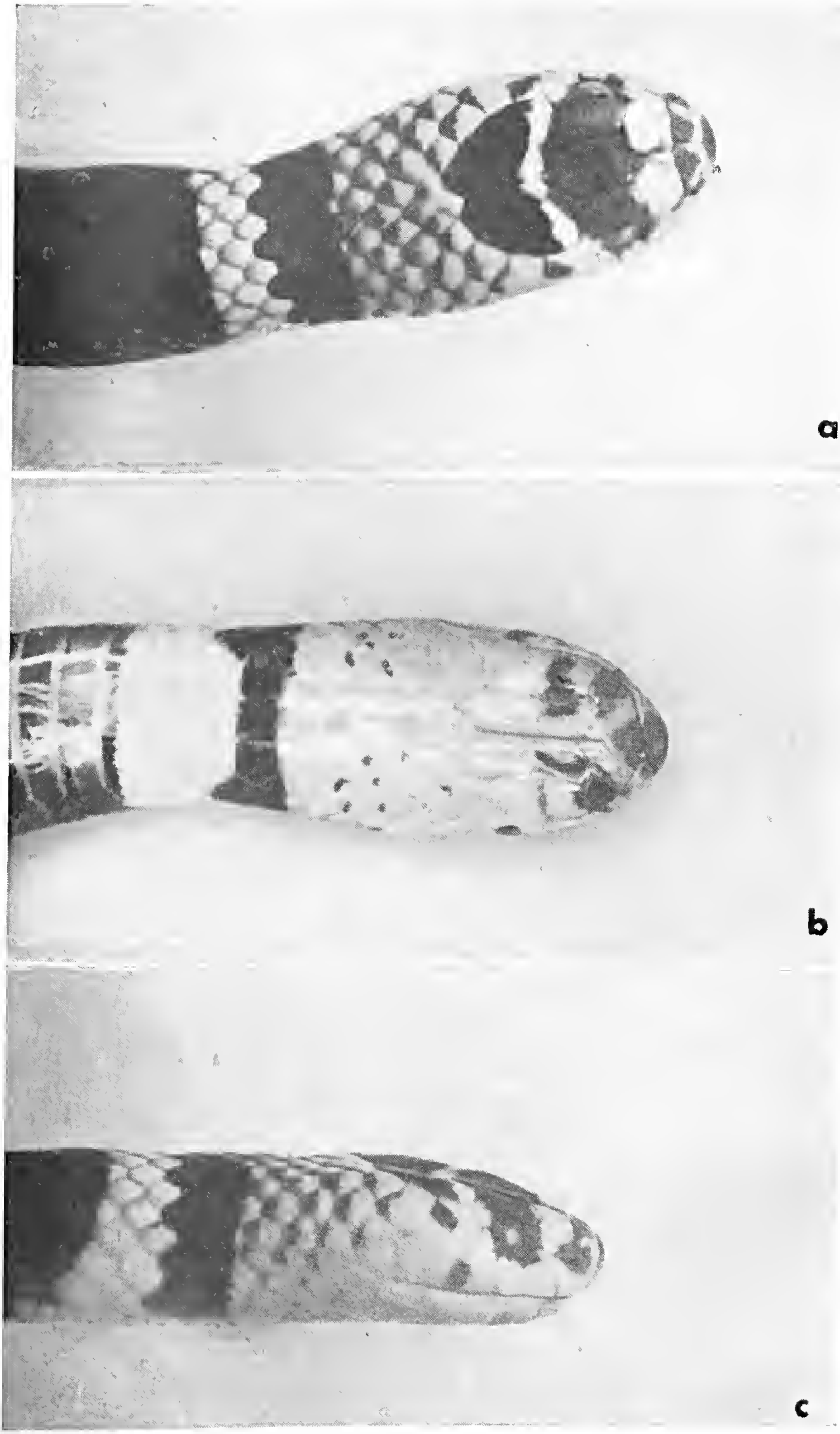

Fig. 7 — Cabeza de *M. f. mesopotamicus.* Paratipo n.º 1627. a) vista dorsal, b) vista ventral, c) vista lateral.

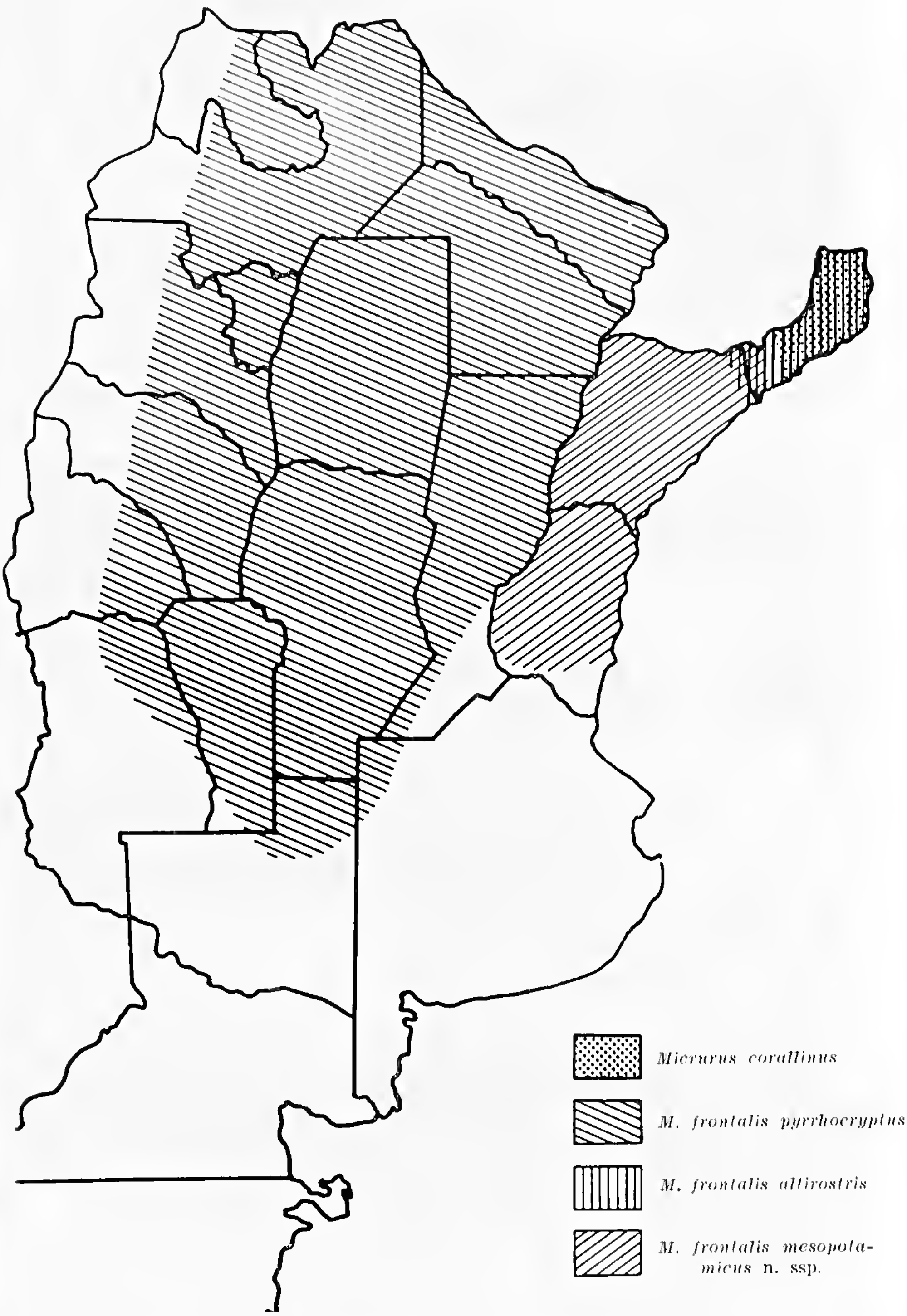

Fig. 8 — Mapa de la distribución de las cuatro entidades del género *Micrurus* en la Argentina.

Colonia Belgrano, Misiones, III-65; N.º 2010, Puerto Esperanza, Misiones, 4-IX-61. MACN: N.º 10038 al 10045, Capitán Meza, Alto Paraná, Paraguay, 1938; N.º 12627-12628, Río Uruguay, a 20 km Puerto Bemberg, Misiones, IX-49; N.º 12645-12646, Río Uruguay, a 20 km Puerto Bemberg, Misiones, III-50.

Summary

The morphology of the four entities of the genus *Micrurus* from Argentina are statistically analyzed and their respective venoms studied from the immunological point of view. One of them belongs to the non questioned species *M. corallinus* and the other three are considered as subspecies of *M. frontalis*. The widest area is occupied by *M. f. pyrrhocryptus* which occurs west of the rivers Paraguay and Paraná; to the northeast, in Misiones Province, *M. corallinus* and *M. frontalis altirostris* overlap. Our new subspecies which we have called *M. f. mesopotamicus* inhabits an intermediate area with natural boundaries given by the rivers Uruguay and Paraná. Morphological characters are also intermediate to those of the other two subspecies, *M. f. pyrrhocryptus* and *M. f. altirostris*. From the immunological point of view each entity shows common and non common precipitins bands to the others, being the divergency more apparent among *M. corallinus* and *M. frontalis* group than among each entity of the latter.

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27. *IN VIVO* NEUTRALIZATION OF CORAL SNAKE VENOM

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INTRODUCTION

Treatment of persons bitten by venomous snakes with antivenin is generally considered one of the most effective forms of therapy. The potency of the particular antivenin usually is based on an *in vitro* neutralization test where venom and antiserum are mixed in a tube, incubated and injected into mice. The results of this type of test largely reflect the degree of neutralization that occurs in the tube. However, in the case of snakebite, and in the subsequent antivenin therapy, the true measure of potency and efficacy of antiserum is more logically based on its capacity to neutralize venom *in vivo*.

The present studies were designed to evaluate the effectiveness, *in vivo*, of coral snake antiserum in neutralizing the lethal effects of coral snake venom. Similar tests were also conducted with other venoms and antisera.

IN VIVO NEUTRALIZATION OF *MICRURUS FULVIUS* VENOM IN MICE BY HOMOLOGOUS ANTISERUM PRODUCED IN GOATS

Neutralization experiments were carried out with a randomly bred strain of albino, male, mice, weighing 16-18 grams. The *Micrurus fulvius* venom used was a freeze-dried pool of numerous milkings of a large number of snakes. The antiserum was a pool of two hyperimmune goat sera (1) specific for *M. fulvius* venom.

The *in vivo* neutralization tests were carried out by injecting groups of mice with venom. Each group of envenomed mice was then injected with goat antiserum at a different time interval. Control mice received normal goat serum following envenoming. Different combinations of routes of injections were used for the venom and antiserum.

The initial group of experiments was conducted with *M. fulvius* venom and anti-*M. fulvius* goat serum. The results of these experiments are shown in Table 1. The intraperitoneal LD_{50} for the venom was 13 μg. All mice received 2 LD_{50} of venom. The antiserum dose was contained in 0.25 ml, and had an *in vitro* neutralization potency of 13.6 mouse LD_{50} per dose. When venom was given intraperitoneally and antiserum was given intravenously, mice were completely protected by antiserum given immediately after the venom and some of the mice were protected when it was given after 30 minutes. Mice were not protected when antiserum was given after 1 hour. However, the time of death for these mice was prolonged 3 to 5 hours beyond the controls. Control mice which received normal serum died between 4 to 6 hours.

When 2 LD_{50} of venom were given subcutaneously and antiserum intravenously, mice were not protected nor were death times prolonged as compared to controls. The subcutaneous LD_{50} of the venom was 32.5 μg which indicated greater tolerance by mice to venom administered by this route than by the intraperitoneal route. This suggests that even though the mouse is more resistant to venom deposited subcutaneously, neutralization of this venom by antiserum given intravenously is not as effective as when the venom was deposited intraperitoneally. To further test this phenomenon, venom was again given subcutaneously but the same antiserum dose was given intraperitoneally. The antiserum failed to protect the mice further confirming the observation that the route of venom injection — in this case subcutaneously — is a factor in the ability of a given dose of antiserum to protect mice *in vivo*. In addition to the route of venom injection, the ability to bring about *in vivo* neutralization by antiserum should depend on the potency and volume of antiserum used. This is illustrated by the last experiment reported in Table I which included antiserum over three times more potent. Protection was obtained when the stronger antiserum was given immediately and some of the mice were protected when it was given after 30 minutes. These results suggest that the routes of envenomation determine the dose of antiserum required for *in vivo* neutralization of the venom.

TABLE I — *IN VIVO* NEUTRALIZATION OF *M. FULVIUS* VENOM IN MICE BY HOMOLOGOUS GOAT ANTISERUM

Route venom injected	Route antiserum injected	Survivors as a function of time antiserum given				Prolongation of death time by antiserum (*Hours*)
		Time		*Alive*	*Dead*	
Intraperitoneal	Intravenous	0 min	Control	0	6	
			Antiserum *	6	0	
		30 min	Control	0	6	
			Antiserum	2	4	3 to 5
		1 hr	Control	0	6	
			Antiserum	0	6	3 to 5
Subcutaneous	Intravenous	0 min	Control	0	6	
			Antiserum *	0	6	0
Subcutaneous	Intraperitoneal	0 min	Control	0	6	
			Antiserum *	0	6	3 to 5
			Antiserum **	5	1	
		30 min	Control	0	6	
			Antiserum *	0	6	
			Antiserum **	2	4	0

* Antiserum dose neutralized 13.6 mouse LD_{50} *in vitro*.
** Antiserum dose neutralized 48.0 mouse LD_{50} *in vitro*.

IN VIVO NEUTRALIZATION OF *M. FULVIUS* VENOM IN MICE BY SÔRO ANTIELAPÍDICO

A similar experiment using Sôro Antielapídico, a purified antivenin produced in horses at the Instituto Butantan, was conducted. Although this serum is produced with venoms from South American coral snakes, it is more than

90% effective in neutralizing *M. fulvius* venom. The results of this experiment are summarized in Table II. The same *M. fulvius* venom dosage and routes of injection used previously were employed. Protection of most mice was obtained if antiserum was given within 30 minutes of envenomation and the time to death was prolonged for several hours when the antiserum was given as late as 1 hour after envenomation. These results are in general agreement with the data from experiments with goat antiserum.

TABLE II — *IN VIVO* NEUTRALIZATION OF *M. FULVIUS* VENOM IN MICE BY SÔRO ANTIELAPÍDICO

Route venom injected	Route antiserum injected	Survivors as a function of time antiserum given				Prolongation of death time by antiserum
		Time		*Alive*	*Dead*	*(Hours)*
Intraperitoneal	Intravenous	0 min	Control	1	5	
			Antiserum *	6	0	
		30 min	Control	1	5	
			Antiserum	4	2	6 to 8
		1 hr	Control	0	6	
			Antiserum	1	5	1 to 3
		2 hr	Control	0	6	
			Antiserum	0	6	0

* Antiserum dose neutralized 16.2 mouse LD_{50} *in vitro*.

IN VIVO NEUTRALIZATION OF *M. FULVIUS* VENOM IN GUINEA PIGS BY HOMOLOGOUS GOAT ANTISERUM

An *in vivo* neutralization experiment was conducted in guinea pigs. The LD_{50} for Hartley, female, 120-130 gram guinea pigs was found to be 23.3 μg by the subcutaneous route. as compared to 32.5 μg for this route in 16-18 gram mice, 30% less venom was required to achieve an LD_{50}. This indicated that these guinea pigs were considerably more sensitive to the venom than the mice and for these two species a toxicity relationship based on a unit of body weight is not valid. The guinea pigs received 2 LD_{50} of venom subcutaneously and the goat antiserum intraperitoneally. The antiserum had a neutralizing potency *in vitro*, of 40.8 mouse LD_{50} per dose. The results of this experiment are shown in Table III. Complete protection was obtained when antiserum was given 30 minutes after envenomation. Antiserum given after 1 hour did not prevent death; however, the time of death was prolonged by as much as 24 hours. Antiserum given as late as 2 hours after envenomation still prolonged the time of death several hours. Control guinea pigs which received normal serum died after 4 hours.

In experiments with both guinea pigs and mice, antiserum gave complete protection only when it was injected within 30 minutes of envenomation. However, the time of death for guinea pigs was prolonged nearly 24 hours longer than in mice even though they both received the same dose of antiserum.

TABLE III — *IN VIVO* NEUTRALIZATION OF *M. FULVIUS* VENOM IN GUINEA PIGS BY HOMOLOGOUS GOAT ANTISERUM

Route venom injected	Route antiserum injected	Survivors as a function of time antiserum given				Prolongation of death time by antiserum (*Hours*)
		Time		*Alive*	*Dead*	
Subcutaneous	Intraperitoneal	0 min	Control	0	3	
			Antiserum *	3	0	
		30 min	Control	0	3	
			Antiserum	3	0	
		1 hr	Control	0	3	
			Antiserum	0	3	24
		2 hr	Control	0	3	
			Antiserum	0	3	2 to 4

* Antiserum dose neutralized 40.8 mouse LD_{50} *in vitro.*

IN VIVO NEUTRALIZATION OF *NAJA ATRA* VENOM IN MICE BY HOMOLOGOUS ANTISERUM PRODUCED IN HORSES

Experiments were designed to determine whether the need to administer antiserum within 30 minutes was a characteristic unique to the coral snake venom system. For this purpose *in vivo* neutralizations were conducted with the venom of *Naja atra*, the Formosan cobra. Crude, horse, anti-*Naja atra* serum was obtained from Cmdr. G. S. Huber, MSC, USN, NAMRU-2, Taipei, Taiwan. The venom and antiserum dosages were the same as those used in previous tests. Results are presented in Table IV. The data are similar to the results obtained with coral snake venom neutralizations. Antiserum must be given within 30 minutes for protection of mice and it failed to prolong death times.

IN VIVO NEUTRALIZATION OF *CROTALUS D. TERRIFICUS* VENOM IN MICE BY HOMOLOGOUS ANTISERUM PRODUCED IN HORSES

To study the *in vivo* neutralization of a crotalid venom mice were given *Crotalus d. terrificus* venom followed by a commercially prepared antivenin * produced in horses with a mixture of four crotalid venoms. This product has wide neutralizing activity for many species of the CROTALIDAE of the Americas. In establishing the dose the antivenin potency was based on its ability to neutralize *Crotalus d. terrificus* venom. The antivenin dosage was adjusted to the same range as those used in the previous studies. The results of this experiments are shown in the lower half of Table IV. Mice were protected only if antivenin was given immediately after envenomation. There was only slight prolongation of death times when antivenin was given after 30 minutes. These neutralizations were slightly less effective than those observed with cobra and coral snake venom.

* Wyeth Laboratories, Philadelphia, Pennsylvania, U.S.A.

TABLE IV — *IN VIVO* NEUTRALIZATION OF *NAJA ATRA* VENOM IN MICE BY HOMOLOGOUS HORSE ANTISERUM

Route venom injected	Route antiserum injected	Survivors as a function of time antiserum given				Prolongation of death time by antiserum (*Hours*)
		Time		*Alive*	*Dead*	
Intraperitoneal	Intravenous	0 min	Control	0	6	
			Antiserum *	6	0	
		30 min	Control	0	6	
			Antiserum	3	3	0

IN VIVO NEUTRALIZATION OF *CROTALUS D. TERRIFICUS* VENOM IN MICE BY CROTALID ANTIVENIN

Route venom injected	Route antiserum injected	Survivors as a function of time antiserum given				Prolongation of death time by antiserum (*Hours*)
		Time		*Alive*	*Dead*	
Intraperitoneal	Intravenous	0 min	Control	0	6	
			Antiserum **	5	1	
		30 min	Control	1	5	
			Antiserum	1	5	2
		1 hr	Control	0	6	
			Antiserum	0	6	1

* Antiserum dose neutralized 13.4 mouse LD_{50} *in vitro*.
** Antiserum dose neutralized 16.2 mouse LD_{50} *in vitro*.

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28. LAS DIFERENTES POBLACIONES DE *BOTHROPS ALTERNATA* DUMÉRIL Y BIBRON (*OPHIDIA, CROTALIDAE*) DE LA ARGENTINA, CONSIDERADAS DESDE EL PUNTO DE VISTA MORFOLÓGICO Y ANTIGÉNICO

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El crotálido *Bothrops alternata* es una especie considerada monotípica y de amplia dispersión que abarca ciertas regiones del sur y centro del Brasil, sur del Paraguay, Uruguay y Argentina. En lo que respecta a su distribución geográfica en la Argentina ocupa una amplia área continua al este del meridiano 64 que se extiende desde el límite septentrional del país hasta el paralelo 34 aproximadamente. A partir de esa latitud aparecen en la provincia de Buenos Aires tres conjuntos poblacionales totalmente desconectados entre sí, tal como uno de nosotros lo hiciera notar en trabajo anterior (1) (Fig. 1).

Hasta el momento no se han señalado subespecies de esta entidad a pesar de su vasta distribución. La comprobación de diferentes patrones de diseño así como posibles diferencias inmunoquímicas de sus venenos nos llevaron a estudiar comparativamente muestras poblacionales tanto desde el punto de vista morfológico-estadístico como inmunológico con el fin de poder establecer la existencia de áreas de prevalencia de determinados caracteres y en caso positivo considerar el nivel de diferenciación alcanzado por estas poblaciones.

Material y métodos

Para nuestro estudio se utilizaron ejemplares de *Bothrops alternata* llegados al Instituto Nacional de Microbiología "Carlos G. Malbrán" desde diferentes puntos del país, los que posteriormente a su extracción de veneno fueron fijados e ingresaron en nuestras colecciones (CHINM). Se investigaron las precipitinas por el método de la doble difusión según Outcherlony (2), Grasset y col. (3) y microdifusión empleado por Schenberg (4, 5). El veneno fue desecado al vacío y guardado separadamente por ejemplar; en el momento de su empleo se diluyó en una proporción de 10 mg/ml de solución fisiológica y se sembraron 0.05 ml en cada cúpula. Se utilizó suero antibotrópico del Instituto Nacional de Microbiología con un título tal que 1 ml de suero neutraliza 2,5 mg de veneno de *Bothrops alternata*, siendo diluído al 50% en solución fisiológica.

Desde el punto de vista morfológico se analizaron estadísticamente los siguientes caracteres: número de escamas dorsales, ventrales, subcaudales y número de diseños básicos de los ejemplares procedentes del área de distribución continua y de cada una de las poblaciones bonaerenses.

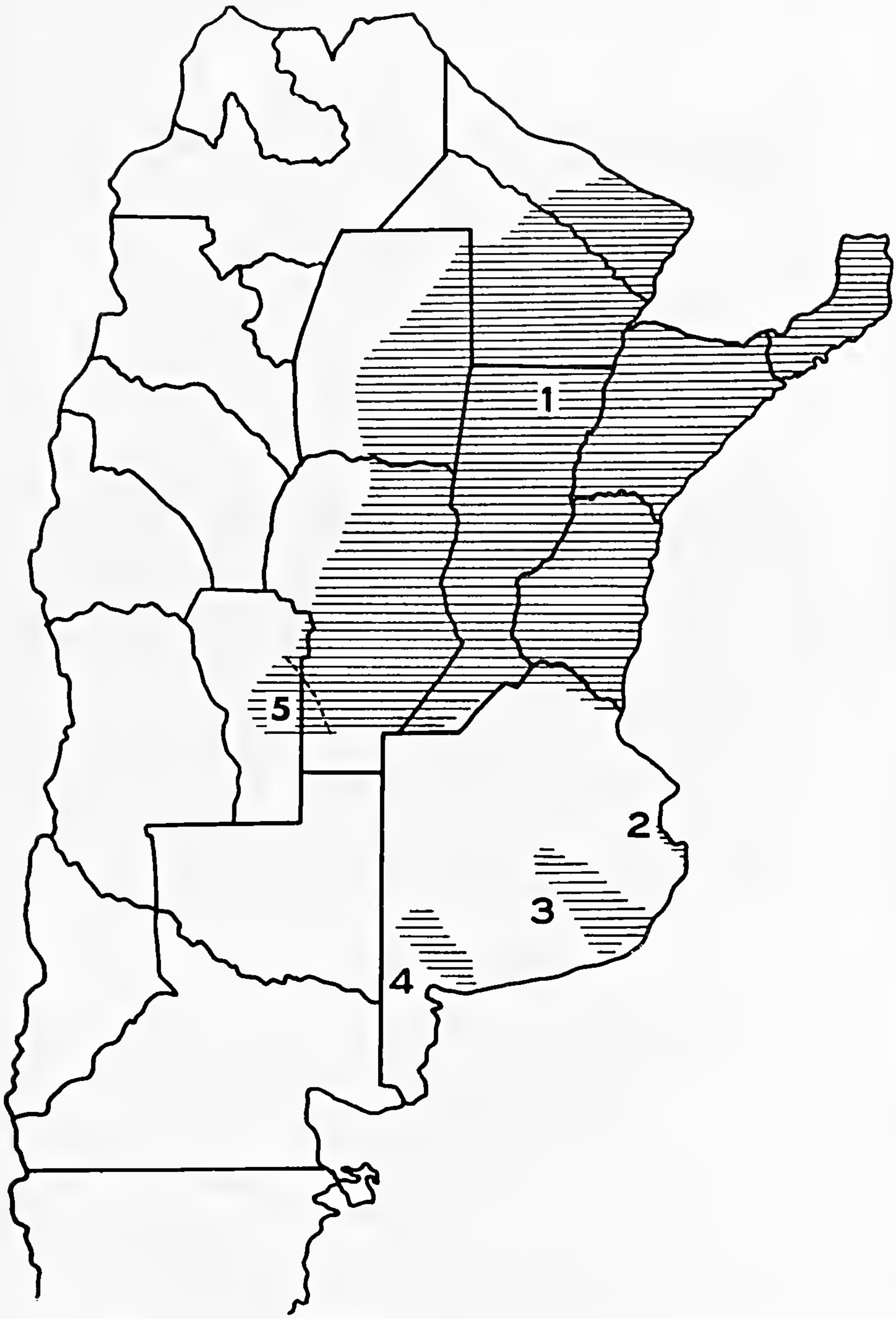

Fig. 1 — Mapa de la distribución geográfica de *Bothrops alternata* en la Argentina. Poblaciones de distribución continua: 1, provincias de San Luis y sudoeste de Córdoba. Poblaciones bonaerenses de distribución discontinua: 2, Gral. Lavalle; 3, Tandilia; 4, Ventania.

Resultados

Análisis inmunológico

El estudio de las precipitinas demuestra que, mientras la diferenciación morfológica es apenas incipiente, como se verá, las variaciones geográficas registrables en la composición antigénica de su veneno son mucho más notables.

Se comprobó que existe homogeneidad dentro de las muestras procedentes del área de distribución continua a pesar de su extensión. No obstante, en el enfrentamiento de muestra de ponzoñas de localidades extremas se ponen de manifiesto ciertas líneas de precipitinas no comunes a la totalidad de ellas. Esta divergencia es mucho más notoria al comparar venenos procedentes de las poblaciones de distribución discontinua de la provincia de Buenos Aires con las del área continua (Fig. 2, 3). También resulta interesante la verificación de la inexistencia de una total identidad inmunoquímica entre las tres poblaciones bonaerenses, aunque debemos notar que la divergencia es menor entre las poblaciones de Tandilia y Ventania que entre cada una de éstas con la de Gral. Lavalle (Fig. 4).

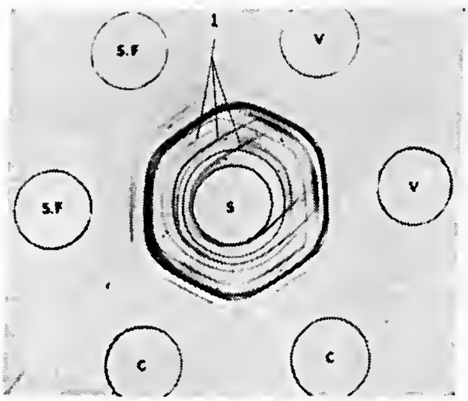

Fig. 2 — Espectro de bandas de precipitinas. S.F: Santa Fe; C: Corrientes; V: Ventania (Bs. As.), S: suero antibotrópico. 1: entrecruzamientos correspondientes a fracciones antigénicas no comunes.

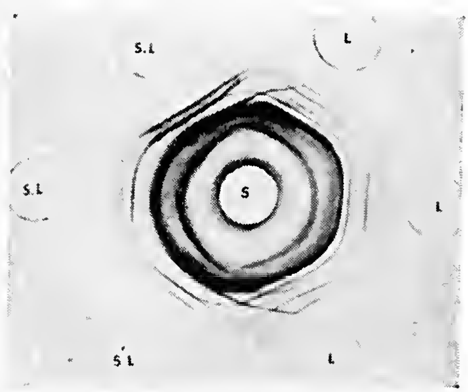

Fig. 3 — Espectro de bandas de precipitinas. S.L: San Luis; L: Gral. Lavalle. (Bs. As.), S: suero antibotrópico.

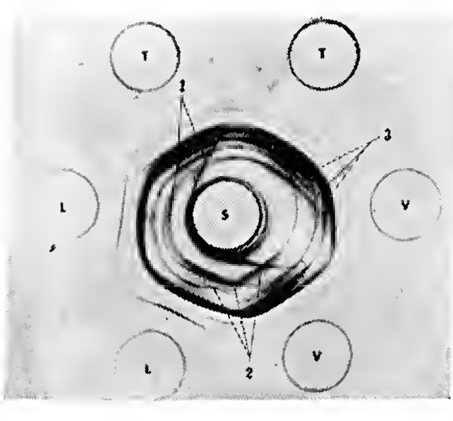

Fig. 4 — Espectro de bandas de precipitinas de las poblaciones de la provincia de Bs. As. V: Ventania; T: Tandilia, L: Gral. Lavalle; S: suero antibotrópico. 1, 2 y 3 entrecruzamientos que demuestran la no total identidad antigénica.

Análisis morfológico

El análisis estadístico nos muestra que en lo referente al número de escamas dorsales, ventrales y subcaudales no hay diferencias apreciables y significativas entre los ejemplares del área de distribución continua y los de las poblaciones de la provincia de Buenos Aires, ni entre estas últimas entre sí (Fig. 7). En cuanto al número de dibujos básicos, si bien el análisis de variancia no permite extraer conclusiones definitivas, parecería existir una tendencia a aumentar su número en el sentido norte-sur. Despecto de la conformación de dicho diseño fundamental también éste varía de norte a sur entre dos patrones extremos, desde la forma arriñonada más característica (Fig. 5) hasta la forma de ∩ con sus dos ramas interrumpidas cerca de su extremo inferior (Fig. 6). Paralelamente a esta modificación se observa una tendencia de los diseños contralaterales a fusionarse en la línea medio dorsal, configurando de este modo un dibujo en forma de X. Por consegniente, de acuerdo a este supuesto se aprecia una mayor incidencia en estos patrones en las poblaciones más meridionales tales como las de las provincias de Buenos Aires. San Luis y sur de Córdoba (Fig. 6).

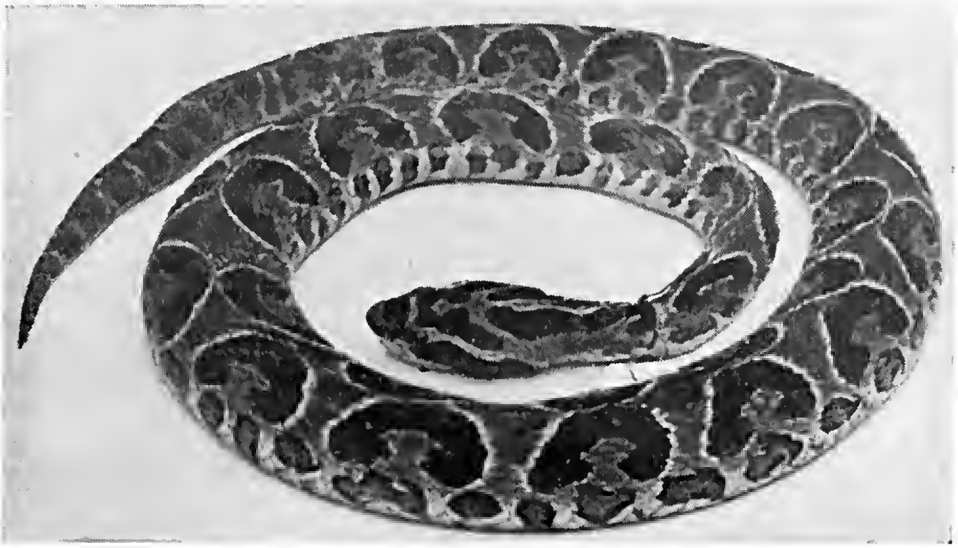

Fig. 5 — *Bothrops alternata.* Procedencia: Sta. Clara de Buena Vista (Santa Fe).

Conclusiones

Los espectros de las precipitinas consignados ponen en evidencia que existe entre los antígenos del veneno una diferenciación inmunoquímica que es más acentuada entre las poblaciones no conectadas en su distribución que entre las colindantes. Diferencias inmunoquímicas interpoblacionales de venenos de *Bothrops neuwiedi* y *Bothrops jararaca* ya fueron señaladas por Schenberg (4. 5).

En cuanto al aspecto morfológico no estamos en condiciones de afirmar categóricamente la existencia de "clines" relativos al número y a las variaciones de los patrones de diseño anotados, aunque los hechos hablarían a favor de tal suposición.

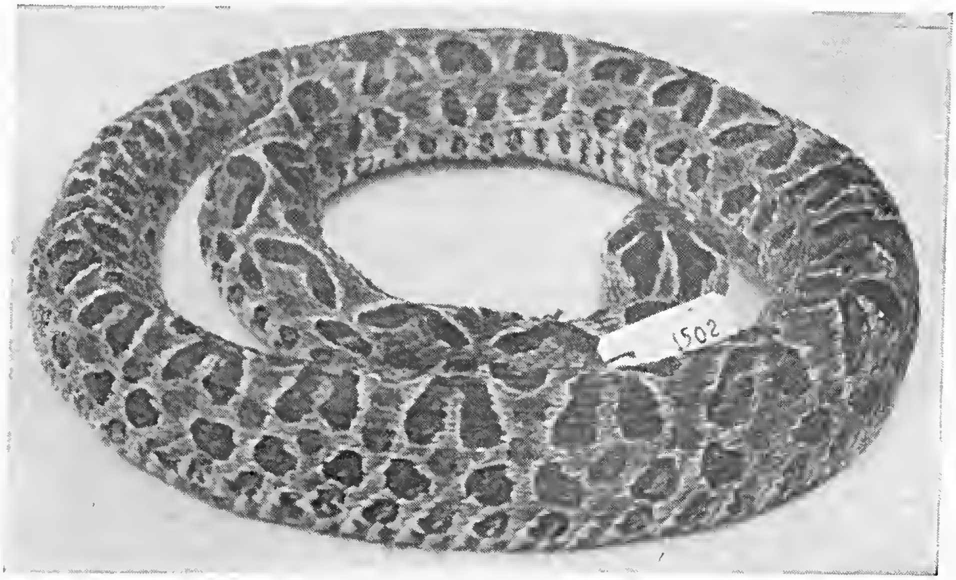

Fig. 6 — *Bothrops alternata*. Procedencia: Sierra de la Ventana. (Buenos Aires).

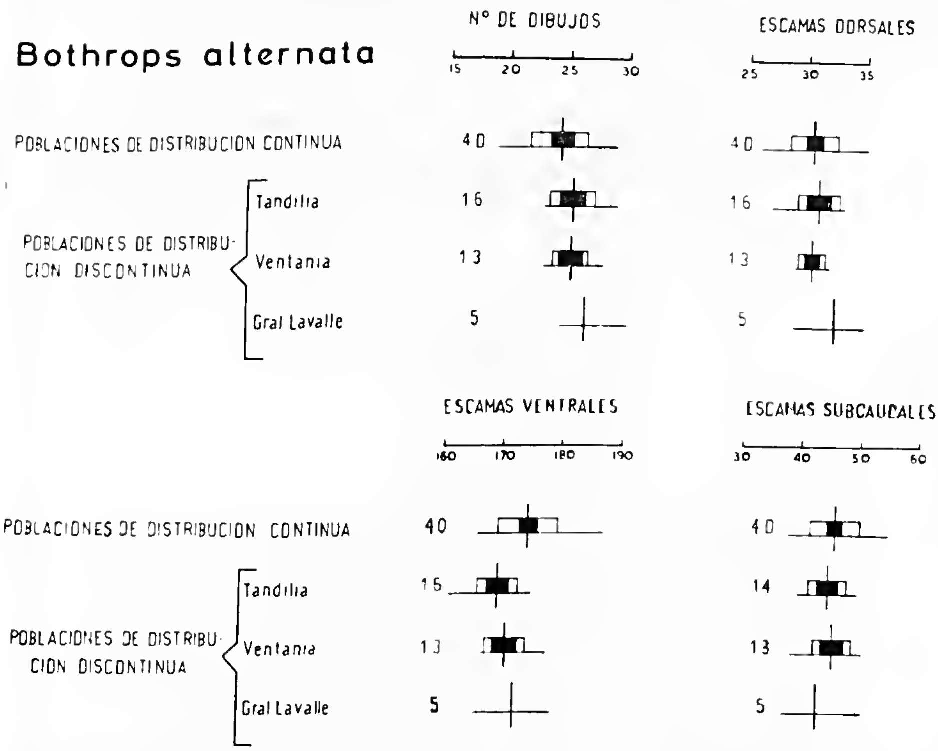

Fig. 7 — Análisis estadístico. La línea horizontal corresponde al rango, la vertical a la media, el rectángulo negro a dos errores standard y el rectángulo blanco a una desviación standard.

También cabe señalar que, dentro de la variabilidad ya comentada, la homogeneidad antigénica de los venenos estudiados es más constante en cada una de las poblaciones que la de los rasgos morfológicos correspondientes. Esto guarda relación con lo ya señalado por diferentes autores (6, 7, 8), en el sentido de que el estudio del veneno de diferentes procedencias aporta datos de indudable valor filogenético al permitir detectar nuevas formas en vías de segregación. Si bien en algunos casos estos cambios se producen paralelamente a la modificación de los caracteres morfológicos, en la mayor parte de las veces la evolución de los componentes químicos de las ponzoñas precede a la de los rasgos morfológicos.

Por todo lo antedicho creemos que estamos ante un proceso evolutivo de iniciación relativamente reciente. Esto puede inferirse de la imprecisa diferenciación morfológica y de la ausencia de áreas netas de predominancia de ciertos caracteres, que evidencian el aún escaso grado de divergencia poblacional. No obstante ello, todo parecía indicar que las poblaciones bonaerenses son relictos fragmentarios de una anterior ocupación subtotal de la provincia de Buenos Aires. Estas, tal vez, estuvieron vinculadas por más tiempo con las del extremo sudoeste del área de distribución continua y, por el contrario, la interrupción del intercambio genético con las poblaciones del litoral parano-platense fue más precoz.

Como conclusión final y siguiendo entre otros a Wilson y Brown (9) e Inger (10), opinamos que las comprobaciones efectuadas no nos autorizan a separar a las diferentes poblaciones bonaerenses de esta especie en entidades de nivel subespecífico.

Summary

The different populations from Argentina of the monotypic crotalid *Bothrops alternata* have been studied from the morphological statistic and immunological point of view. Morphologically, a tendency to increase the number of basical designs and to modify their characteristic conformation from North to South has been observed. This is especially noticeable on the three populations from Buenos Aires and in the southwestern extreme of the continuous distribution area. From the point of view of the antigenic composition of the venoms, immunochemical analysis evidences greater differences between distant populations than between the neighbouring ones. There was not a complete identity among the spectrum of precipitin bands of the three populations from Buenos Aires. The reported facts do not allow us to make a separation of the diverse populations of this species into subspecific entities.

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29. ANTIVENIN PRODUCTION

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Introduction

The production of antivenins in Iran was beset with many difficulties. Since the date of preparation of this serum at Razi Institute, continued efforts have been made to improve the therapeutic properties of the product. From 1960 till now a great number of poisonous snakes from various localities of Iran have been made to improve the therapeutie properties of the product. From month for venom. The venoms are lyophilized and kept for hyperimmunization and titration. Razi Institute produces the following antivenins:

a) Monovalent antivenins against the venom of:

Naja naja, Vipera lebetina, Vipera xanthina, Vipera persica persica, Echis carinatus, Agkistrodon halys.

b) Polyvalent antivenin against the venom of:

Naja naja, Vipera lebetina, Vipera xanthina, Vipera persica persica, Echis carinatus, Vipera ammodytes, Agkistrodon halys.

c) Polyvalent antivenin against the venom of:

Naja naja, Bungarus fasciatus (Krait), *Echis carinatus, Vipera russelli.*

All antivenins are produced as pepsin digested. ammonium sulphate concentrated solution containing 10-18 gr per cent protein.

Materials and Methods

We produce our antivenins according to methods used in Butantan Institute, São Paulo. Brazil. Production begun as early as 1960 and serum was issued for therapeutic use in 1961. The snakes are obtained by purchase from people who catch them and bring them to the laboratory. The snakes are milked once a month for venoms. For obtaining the venom from the poisonous glands in *Naja*, the fangs are inserted on the rubber of the dishes and in VIPERIDAE the fangs are pressed directly inside the dishes down to the glass. The venom is refrigerated at —18°C. for 24 hours and dried in dessicator under vaccum over P_2O_5. The dried venom is kept away from light and humidity. For immunization as well as serum titration we always employ a pool of dried venom from several batches for preparing our stock. The dried venom is weighted

very carefully in an analytical balance. A ten per cent solution is prepared for hyperimmunization using 50% sterile physiological salt and glycerin with a piece of camphor, as stock solution. To determine the CLD, LD_{50} and serum potency titration, one per cent solution is prepared using sterile physiological salt as the initial dilution; determinations were made in white mice weighing 16-18 gr through the intravenous route.

For the production of the antivenin, healthy horses more than five years old (usually 7-8) are selected. Observations indicate that very young and old horses do not respond very well to immunization procedures. The general scheme followed in the immunization of horses is to gradually build up their antivenin titre by the regular subcutaneous injection of increasing doses of the unmodified venoms employed for hyperimmunization which is prepared by diluting the stock solution as already mentioned. According to our observations, it is better to start the injections of the horses with a small amount of venom as indicated in Table I, to avoid producing toxic symptoms and also to get a higher titre in polyvalent antivenins. The range of the mixture of venoms is shown in Table II; the initial dose contained the equivalent of 0,1 mg in 10 ml of volume, the injections were given twice a week up to the amount of 10 mg, then were followed weekly in volume of 20 ml intervals, the highest amount of injected venom is 120 mg; Re-immunization began with 2 mg of unmodified venom. During the period of immunization three injections of one ml sterile oil, as adjuvant is sufficient. The oil is prepared for adjuvant using 75 gr arabic white gum, 300 ml olive oil, by distilled water up to 600 ml total volume, issued and autoclaved.

TABLE I — PROTOCOL OF IMMUNIZATION SCHEDULE OF HORSES

Period of injection	Antigen		Administration
	Venom mg.	Total volume with saline	
1	0,1	10 ml	Subcutaneous
2	0,2	10 ml	Subcutaneous
3	0,4	10 ml	Subcutaneous
4	0,7	10 ml	Subcutaneous
5	1	10 ml	Subcutaneous
6	2	10 ml	Subcutaneous
7	4	10 ml	Subcutaneous
8	7	10 ml	Subcutaneous
9	10	+ 1 ml oil = 20 ml	Subcutaneous
10	20	20 ml	Subcutaneous
11	30	+ 1 ml oil = 20 ml	Subcutaneous
12	40	20 ml	Subcutaneous
13	60	+ 1 ml oil = 20 ml	Subcutaneous
14	80		Subcutaneous
15	100	20 ml	Subcutaneous
bleeding			
bleeding			
bleeding	6 to 8 lit. for each horse		

TABLE II — RANGE OF POLYVALENT MIXTURE OF VENOMS EACH 1/12 EQUIVALENT TO 200 MG DRY VENOM IN 2 ML SOLUTION

Venoms	P_1	P_2
Naja naja	3/12	4/12
Vipera lebetina	3/12	1/12
Echis carinatus	2/12	3/12
Vipera xanthina	1/12	—
Vipera persica persica	1/12	—
Vipera ammodytes	1/12	—
Agkistrodon halys	1/12	—
Vipera russelli	—	2/12
Bungarus fasciatus (Krait)	—	2/12

Purification and concentration of antivenin

Methods devised for the purification and concentration of antitoxins have been applicable to antivenins also. The technique adapted in this laboratory is that originated by Pope and developed by Grasset and Christensen (1947), and Delsal and Mirchamsy (1953). The routine procedures followed in Razi Institute are outlined below.

The immune horses are bled into Sodium citrate. Six to eight liters of blood are removed from each horse on each of the three occasions in three days. The plasma is separated from the red cells by siphon, and sufficient phenolether mixture is added to give a phenol concentration of 0.5 per cent. 100 litres of plasma are mixed with 200 litres of Saline and the mixture is adjusted by pH 3.2, pepsin (1/10000 titre) is added to 0.5 gr per cent of plasma and the reaction is brought to pH 3.2. After 30 minutes, digestion at 30°C. 0.2 per cent of trisodium phosphate, 0.1 per cent of toluine, 14 per cent of ammonium sulphate are added to the mixture, while being stirred mechanically. The reaction is brought to pH 5.2, the mixture is steam-heated rapidly to 55°C and maintained at this temperature for 60 minutes. At the end of the heating period the mixture is water-cooled and the denatured protein is removed by filtration through filter cloth under slight positive pressure. The filtrate is added 0.2 per cent of trisodium phosphate and adjusted to pH 7.4, with 17 per cent of ammonium sulphate. Antibody-earrying protein precipitate is removed by filtration through filter cloth, pressed, weighted, dialized for 48 hours in running water. The volume of the dialized solution is determined and 40 per cent of aluminium gel is added (Aluminium Gel prepared at Willstater technique). Then it is heated at 50°C. for 60 minutes; the gel is removed by filtration or centrifugation and the requisite amounts of salt and phenol are added. pH 6.8-7.2, phenol concentration 0.35 per cent, protein 10-18 per cent, albumine negative. The solution of refined globulins is sterilized by filtration, checked for pH, protein, electrophoreses, phenol, sterility, innocuity, pyrogenicity and potency and ampouled for issue. The final results of polyvalent antivenin are a recovery 37-65 (52%), purification indices 1.1-2.39 (main 1.76), concentration indices 1.30-2.7 (main 1.85). The titration is made by different methods, but the

routine procedure is outlined below. Different amounts of pooled standard venom are added to a series of haemolytic tubes containing 0.1 ml of serum, total volume 0.2 ml is injected intravenously to white mice weighting 16-18 gr. Each dilution is injected to five mice and neutralization is calculated as mg weight of dry venom neutralized one ml of serum. For example the potency test of polyvalent antivenin for two batches as indicated in Tables III and IV.

TABLE III — TITRE OF PLASMA AND SERUM AGAINST VARIOUS SNAKE VENOMS OBTAINED IN IRAN. THE TITRE ARE GIVEN IN MG. VENOM NEUTRALIZED BY 1 ML OF PLASMA OR CONCENTRATED SERUM. THE M.L.D. OF VENOMS IN MG

Antivenin issued for Iran	*Naja naja*	*Vipera lebetina*	*Vipera xanthina*	*Vipera persica*	*Echis carinatus*	*Vipera ammodytes*	*Agkist. halys*
Polyvalent P_1							
Plasma	0.2	0.8	0.2	0.4	0.5	0.3	0.3
Refined Serum	0.6	2.2	1	1.4	1.7	1.2	4.4
M.L.D.	0.009	0.010	0.007	0.028	0.004	0.010	0.014
Monovalent							
Plasma	0.6	1.5	0.4	0.6	0.6	0.8	0.6
Refined Serum	0.8	3	1.6	2.4	1	1.2	0.8

TABLE IV — TITRE OF PLASMA AND SERUM AGAINST THE VENOMS OF FOREIGN ORIGIN

Antivenin issued for neighboring countries	*Naja naja*	*Bung. fasc.* (Krait)	*Echis carinatus*	*Vipera russelli*	*Vipera lebetina*
Polyvalent P_2					
Plasma	0.2	0.8	1.2	0.5	0.4
Refined Serum	0.6	1	1.6	1.2	1.6
M.L.D.	0.009	0.020	0.015	0.020	0.010

Acknowledgement — The authors are grateful to Prof. Dr. M. Kaveh, General Director of Razi Institute for his encouragement and for providing us the materials needed for this work; Dr. H. Mirchamsy, Assistant Director, for his useful data and comments. We wish to extend our thanks to Prof. Dr. A. Rafyi, Dean of the Veterinary College-Iran, for his encouragement and valuable help and interest. Also we are grateful to Prof. Dr. R. Furlanetto, Head of the Dept. of Immunology of Butantan Institute — Brazil, for his continuous interest in our work and his kind attention. We are thankful and indebted to the following colleagues: Dr. S. Korourian, Dr. F. Nazari, Dr. M. Eliazan, and Dr. R. Farzanpy for their help and assistance.

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IV

BIOQUÍMICA

BIOCHEMISTRY

30. SEPARATION OF TOXIC COMPONENTS FROM THE BRAZILIAN SCORPION — *TITYUS SERRULATUS* — VENOM

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Separation of protein components from *T. serrulatus* venom has already been made using mainly electrophoresis techniques (1). In the present paper results will be reported on the separation of toxic components from the venom of *T. serrulatus* by a combination of extraction and chromatographic techniques using dextran gels and the resin carboxymethylcellulose. The procedure used allows the separation of a toxic component in highly purified form. Columns of 29 × 2.5 cm were packed with the fine grade suspension of Sephadex G-25 in distilled water, following the instructions of Gelotte (2). CM-cellulose was used in 0.6 × 22 cm columns prepared according to Peterson and Sober (3). Buffers were prepared from analytical grade reagents dissolved in glass distilled water. Scorpion venom obtained by electrical stimulation was supplied by Instituto Butantan and was used in all the experiments. Cellulose acetate paper electrophoresis was run according to Kohn (4). Determinations of the LD_{50} (5) were made by the intraperitoneal route in mice.

Results

Venom obtained by electrical excitation contained insoluble substances that were separated by successive cold water extractions. The water soluble extract was chromatographed on Sephadex G-25 columns using water and 0.1 TRIS buffer or 0.1 M ammonium acetate as eluents. Two main protein peaks P_1 and T_1 (Fig. 1) appeared in the water eluates. Component T_1 contained approximately 30% of the toxic activity evaluated by injection in mice. TRIS buffer eluted two additional components P_2 and T_2 (Fig. 1), the bulk of toxicity being found in peak T_2. Elution with ammonium acetate following water elution (Fig. 2), showed a small inactive protein peak and a large peak where the toxic activity was found. Electrophoresis on cellulose acetate paper revealed that peak T_2 obtained either with TRIS or acetate buffers still contained several components. For further purification elution with ammonium acetate was chosen and

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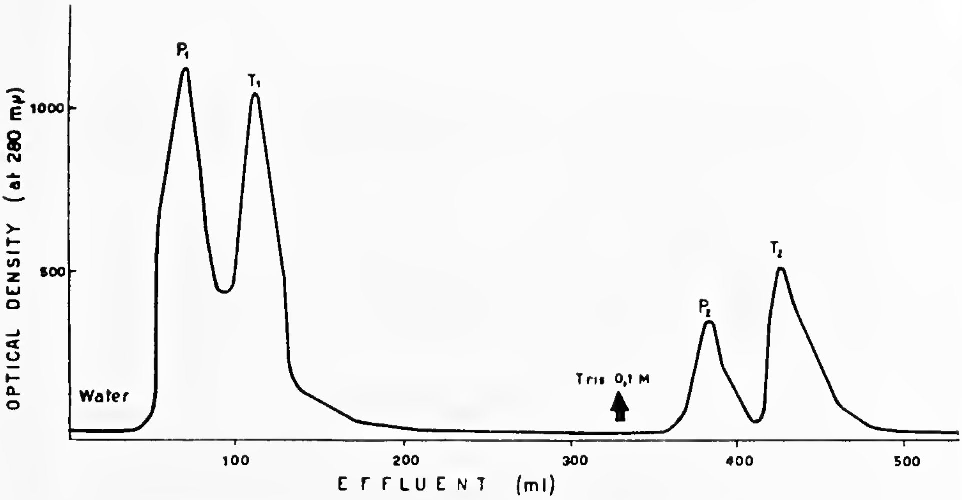

Fig. 1 — Chromatogram of 100 mg of total venom on a column of Sephadex G-25, 29 × 2.5 cm Flow rate 30 ml/h. Stepwise elution with water and Tris 0.1 M.

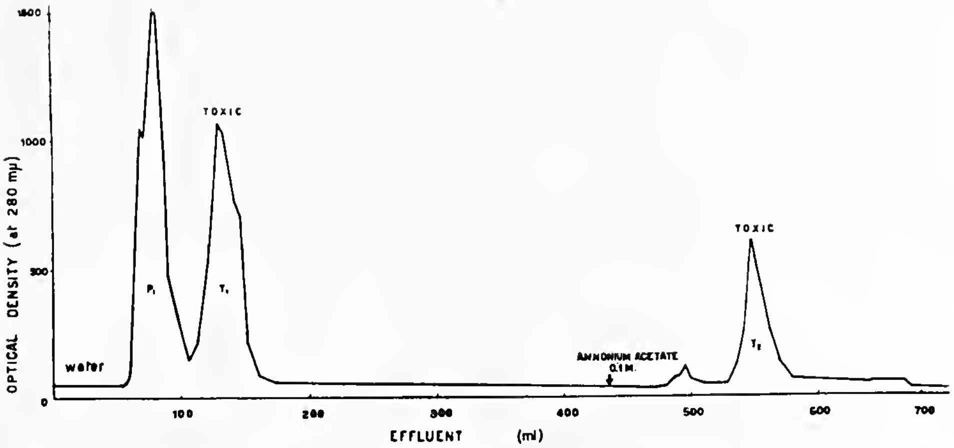

Fig. 2 — Chromatogram of 80 mg of total venom on a column of Sephadex G-25, 37 × 2.5 cm Flow rate 30 ml/h. Stepwise elution with water and ammonium acetate 0.1 M.

the T_2 component was lyophylized and suspended in 0.01 M ammonium acetate pH 7.7. An inactive precipitate formed and was removed by centrifugation. The soluble supernatant was transfered to the CM-cellulose column and eluted with 0.01 M ammonium acetate pH 7.7. Several inactive protein peaks appeared but the toxic material was retained. Following several attempts the elution of the toxic fraction could be achieved by 0.15 M ammonium acetate adjusted at pH 9.0 (Fig. 3).

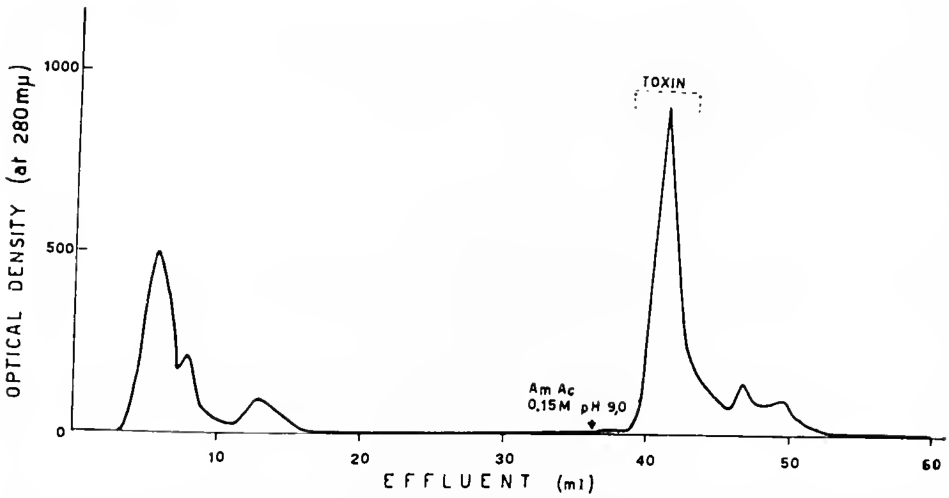

Fig. 3 — CM-cellulose column 0.6 × 22 cm. Chromatogram of 5.5 mg T_2 obtained by gel filtration. Stepwise elution. Starting buffer: Ammonium Acetate 0.01 M, pH 7.7. Flow rate 1.5 ml/h.

The main toxic peak behaved as a single component when submitted to paper electrophoresis or rechromatography. Specific activities and yields of the toxic component in the various steps are shown in Table I.

TABLE I — PURIFICATION OF A TOXIC COMPONENT FROM *T. SERRULATUS* VENOM

Fractions	Protein mg	LD_{50} μg	Toxic units μg	Specific activity	Yield %
1) Total venom	205,0	65	3.150	15,3	100
2) Aqueous extract	136,0	43,4	3.140	23,0	99,5
3) Sephadex G-25	26,9	20,6	1.300	48,5	41,5
4) C.M.-cellulose	7,5	8,6	870	116,2	27,5

Discussion

The procedure now reported to separate the protein components from the venom of *T. serrulatus* may be useful for preparative purposes. The toxic activity, which was assayed on mice by LD_{50} measurements and by observing intoxication symptoms, particularly salivation and lacrimation, was resolved in two components on Sephadex G-25. Further investigations are necessary to interpret this finding as meaning the presence of at least two toxins in the venom. Chromatography on CM-cellulose of the main toxic component from the Sephadex columns, disclosed non toxic components and led to the preparation of a highly purified toxin, which is homogeneous on paper electrophoresis at different pHs.

Miranda, Rochat and Lissitzky (6), used dextrangels of low porosity as a step in the purification of scorpiotoxins. The usefulness of this procedure is confirmed in our work and, as it is pointed out by these authors, the combination of slight ion exchange and adsorption properties of the gel (2) are responsible for the separation of the basic proteins of the venom.

Summary

A procedure is presented for the separation of a highly purified toxic component from the venom of *T. serrulatus*.

Acknowledgment — One of us (C.R.D.) wish to thank the Faculdade de Medicina de Ribeirão Prêto, U.S.P., the permission to work in this Department.

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31. ON THE TOXIN OF *TRITURUS MARMORATUS* LATR.

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Introduction

The range of the newt *Triturus marmoratus* includes Portugal, Spain and the southern parts of France. The amphibian is green with black speckles above and grey to brown with black or white dots beneath. It reaches a length of 16 cm and a weight of 10 g. Its toxin is supposed to be one of the most active newt toxins with a DLM of 1.8 mg/g white mouse (s.e.) (1). Since no chemical datas were available we studied some chemical and biochemical properties and compared them to the properties of other toxins.

Material and methods

Toxin: The newts were rinsed with destilled water, placed in a beaker and treated with 0.2 ml ether. The ether stimulated the excretion of the skin secretion. The product so obtained forms a characteristically smelling foam which was dissolved in dest. water and freeze-dried. A well fed mature newt yields 20-50 mg dry material. The first milking yielded entirely soluble products. After repeated milking the solubility of the toxin decreased.

For better comparison with other amphibian toxins this method was prefered to obtain the toxin by squeezing the paratoid glands.

Gel-chromatography: Sephadex G-200 was used in a column 80 × 2 cm. The void volume of 85 ml was determinated with blue Dextrane. 5 ml of a solution containing 1 per cent newt venom and 0.9 per cent sodium chloride was placed on the column. The elution was performed with saline. 2 ml fractions were taken.

Gel-electrophoresis: A disc electrophoresis system has been used (2). The small pore gel was prepared by dissolving 750 mg acrylamide (Fluka P 50135), 20 mg N,N'-methylene-bisacrylamide (Fluka P 55626), 0.06 ml 10 per cent solution of N,N,N',N'-tetramethylenethylenediamine (TEMED, Fluka 57580) in 10 ml TRIS-HCl buffer pH 8.9. After deaeration the polymerization was started with 7 mg ammonium persulfate. Glass tubes (10 × 0,5 cm) were filled with 1.2 ml solution to a level of 6 cm and allowed to polymerize under a layer of destilled water.

A large pore gel solution was prepared by dissolving 250 mg acrylamide, 6.2 mg N,N'-methylenebisacrylamide, 0.17 ml 10 per cent TEMED in 10 ml TRIS-HCl buffer pH 6.7, and starting with 3.5 mg ammonium persulfate. 0.2 ml of

this solution were placed on top of the small pore gel and polymerized again under a layer of dest. water. After the polymerization is complete the water is replaced by 20-50 μl of a 1 per cent solution of the toxin in TRIS-HCl buffer pH 6.7. After 90 min a sufficient amount of the sample was soaked into the large pore gel. The rest was removed.

Electrophoresis, staining and destaining has been done as described before (2, 3).

Hemolysis and enzyme assay: These determinations were done as described before (4, 5, 6, 7). Surface tension was measured stalagmometrically.

Results and discussion

The enzyme activities of the *Triturus marmoratus* toxin are similar to those of other amphibians (5, 6). E.g. the phosphatase (Fig. 1) is an unspecific phosphomonoesterase similar to acid prostatic phosphomonoesterase. The maximum of activity is at pH 5.0 (p-nitrophenylphosphate). An amylase similar to unk amylase could be detected (5). The arylamidase has an optimum of activity at pH 7.0-7.5 (L-leucine-β-naphthylamide). It hydrolyses (Table I) the β-naphthylamides of alanine, leucine, methionine and proline at a high rate. The hydrolysis rates of the β-naphthylamides of valine, glycine, lysine, arginine, isoleucine and cysteine decreases in the given sequence down to some per cents of

TABLE I — HYDROLYSIS OF L-AMINO ACID β-NAPHTHYLAMIDES (THE VALUE FOR L-LEUCINE β-NAPHTHYLAMIDE IS GIVEN AS 100)

Bombina species		*Triturus cristatus*		*Triturus marmoratus*	
		ala		ala	
leu	100	met	100	met	100
		pro		pro	
ala		leu	100	leu	100
gly		arg	95	val	80
met	90	val	50	hpro	55
pro		hpro	45	ser	40
		try	40		
lys	45				
arg	40				
his	20				
		gly		gly	
ileu	10	ser	30	his	15
				thr	
glu	5	thr	25	lys	10
asp		glu		arg	
cys	2	his	10	asp	
ser		tyr		cys	
				glu	10
		asp		ileu	
		ileu	10	try	
		cys		tyr	

the first group. The sequence of the hydrolysis rates of *Triturus cristatus* toxin differs from *T. marmoratus* toxin mostly in respect to the β-naphthylamides of lysine and arginine. The first mentioned toxin hydrolyzes these substrates much more readily than the latter. The natural substrates of this enzyme are of course not β-naphthylamides but peptides, it would be probably more correct to term this enzyme as peptidase.

Triturus marmoratus toxin hemolyses human erythrocytes down to dilutions of more than 1:10 millions. In this respect it is markedly more active than the toxins of *T. cristatus* or *Bombina variegata*. The hemolysing principle is considered to be a direct hemolysing protein by the same reasons (e.g. no phospholipase activity, inactivation by proteolytic enzymes) as discussed in a previous paper (6). Such proteins occur in snake venoms (8). In bee venom the direct hemolysing factor is a polypeptide with 26 amino acids (9). In both cases the factor was accompanied by phospholipase. The factors themselves were strongly basic.

In disc electrophoresis the hemolytic protein of *Triturus marmoratus* toxin migrates at pH 8.9 with appr. 80 per cent of the mobility of bromphenolblue (Fig. 2), it is therefore an acidic reacting substance. From Sephadex G-200 it is eluted by 1.9 times the void volume (Fig. 1). This corresponds an apparent molecular size of appr. 40.000. Similar data have been found for *Triturus cristatus* and *Bombina* toxins (6). *Triturus* toxins lower the surface tension markedly more than other proteins of similar mole size and electrophoretic behaviour (1). However, since the hemolytic activity of surface active substances does not always parallel the influence on the surface tension this is not necessary the only explanation for the strong hemolytic action of the *Triturus* toxin.

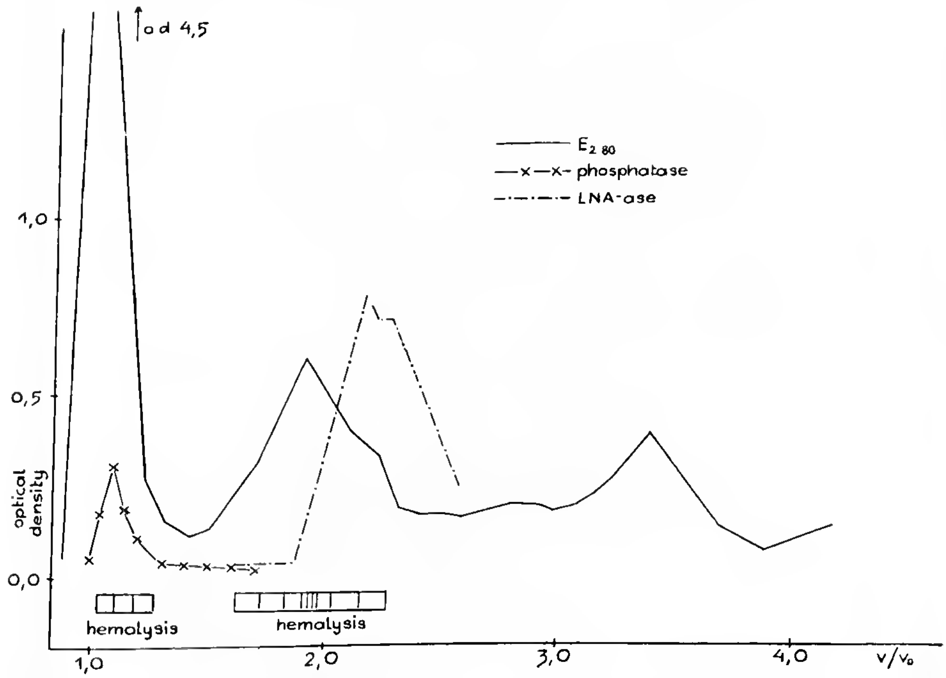

Fig. 1 — *Triturus marmoratus* — Chromatography on Sephadex G-200.

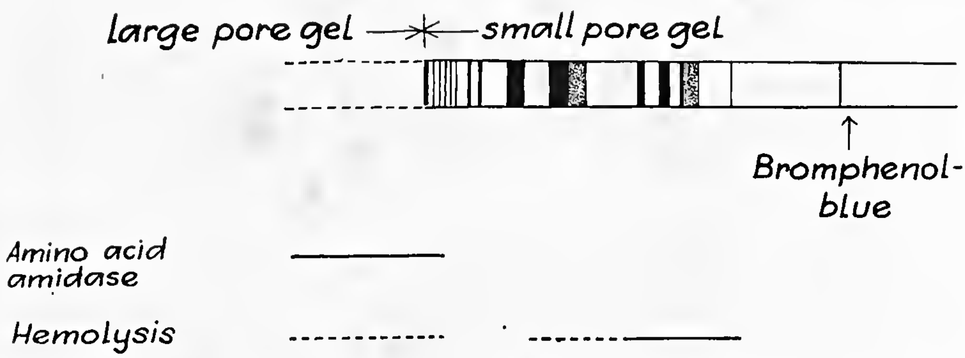

Fig. 2 — *Triturus marmoratus* — Electrophoresis in polyacrylamide.

Summary

Amylase, acidic phosphomonoesterase, arylamidase and a direct hemolysing protein were demonstrated in the toxin of *Triturus marmoratus* Latr. The enzymes and the hemolytic factor were characterized by chromatography on Sephadex, gel electrophoresis and substrate specificity.

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32. BIOCHEMICAL INVESTIGATIONS ON *HELODERMA* VENOM

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Introduction

Beside the snakes the reptiles contain only one venomous lizard family, the *Gila monster*, HELODERMATIDAE. They exist only in two species, the North American *Gila monster*,, *Heloderma suspectum* and its Mexican counterpart, *Heloderma horridum*. In contrary to the snakes their venom glands are located in the lower jaw, the ducts leading to the exterior terminate between the fangs and the lower lip. During the bite, the venom mixed with saliva is conducted into the wound by capillarity. Each fang possesses an indistinct groove along which the venom moves.

The present work deals with the investigation of the venom with modern biochemical and pharmacological methods.

Methods and Material

For the investigations lyophilized venom of *Heloderma suspectum* and *Heloderma horridum* from the MIAMI SERPENTARIUM, Miami, U.S.A., was used. The LD_{50} was determined in mice by subcutaneous injection. The hyaluronidase activity was estimated after the turbidimetric method of DORFMAN and OTT with hyaluronic acid as substrate and compared with a standard preparation of hyaluronidase from ovine testes (500 IU/mg, SERVA, Heidelberg, Germany), the phospholipase A activity was determined by the Marinetti method, the proteolytic activity, according Kunitz, and the kinin-releasing activity from plasma globulines was tested on the isolated guinea pig ileum.

Results and Discussion

The venom of *Heloderma suspectum* and *Heloderma horridum* consists, for the most part, of macromolecular proteins, which can be precipitated with trichloracetic acid and ammonium sulfate.

After 48 hours dialysis against destilled water none of the venom had passed the dialysis membrane. By spectroanalysis it was possible to find potassium, sodium, calcium, magnesium as well as traces of aluminium, zinc, cooper, iron and silicon.

The LD_{50} for *Heloderma suspectum* was 0.82 $\mu g/g$ mouse, for *Heloderma horridum* 1.4 $\mu g/g$ mouse, s.c. injection. The toxic factor is heat stable; heating the venom at pH 7.2, 100°C for 15 min no loss of toxicity was observed.

A comparative estimation of cobra venom toxicity (*Naja naja*) resulted in a LD_{50} of 0.8 $\mu g/g$ mouse, thus in effect the toxicity of *Heloderma* venom is comparable with that of cobra venom.

An enzyme analysis of *Heloderma* venom was run for phosphomono- and diesterase, acetylcholinesterase, nucleotidase, ATPase, DNAase and RNAase, aminoacid oxidase, fibrinogenocoagulase, protease, phospholipase A and hyaluronidase. Of all these enzymes we succeeded only in finding phospholipase A, protease and hyaluronidase. A direct hemolytic factor for example as one can find in small amounts in cobra venom could not be proved, washed human erythrocytes were not hemolysed by *Heloderma* venom.

The hyaluronidase in *Heloderma* venom showed a very high activity and was 20-40 fold higher than that of the ovine testes preparation, for 1 mg *Heloderma* venom 10-20.000 IU could be calculated (Fig. 1).

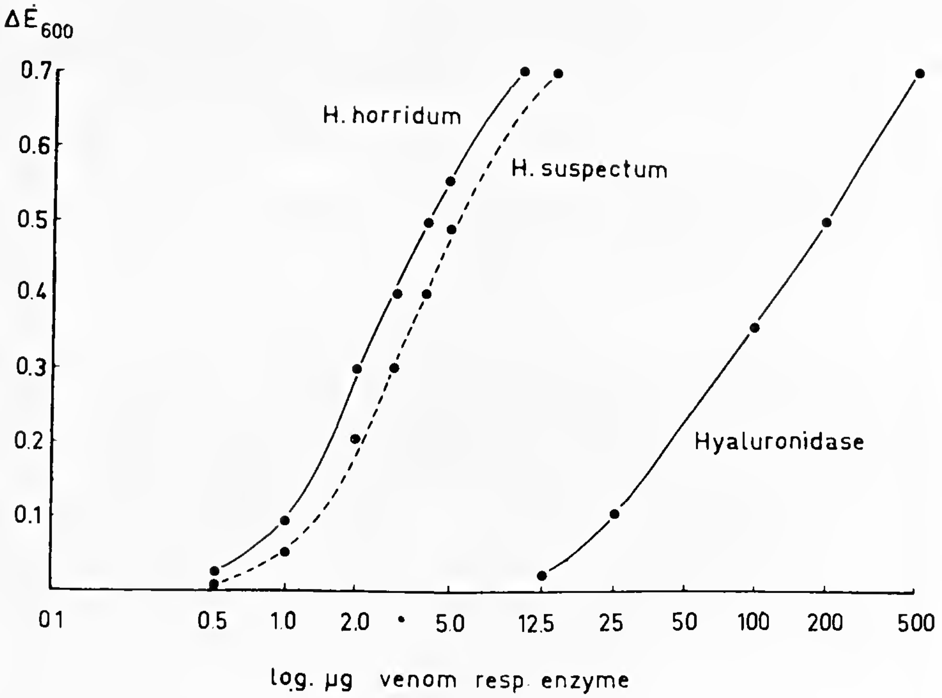

Fig. 1 — Hyaluronidase activity of *Heloderma* venom and Hyaluronidase from ovine testes, in dependence on the turbidity of unaffected hyaluronic acid after incubationtime for 1 h, 37°C.

The phospholipase A which is contained in many animal venoms could be also detected in *Heloderma* venom. Fig. 2 shows the enzyme activity of *Heloderma* venom measured on the rate of clearing of an egg yolk emulsion at pH 7.2, 25°C under the influence of 200 μg venom each, in comparison with cobra venom (*Naja naja*).

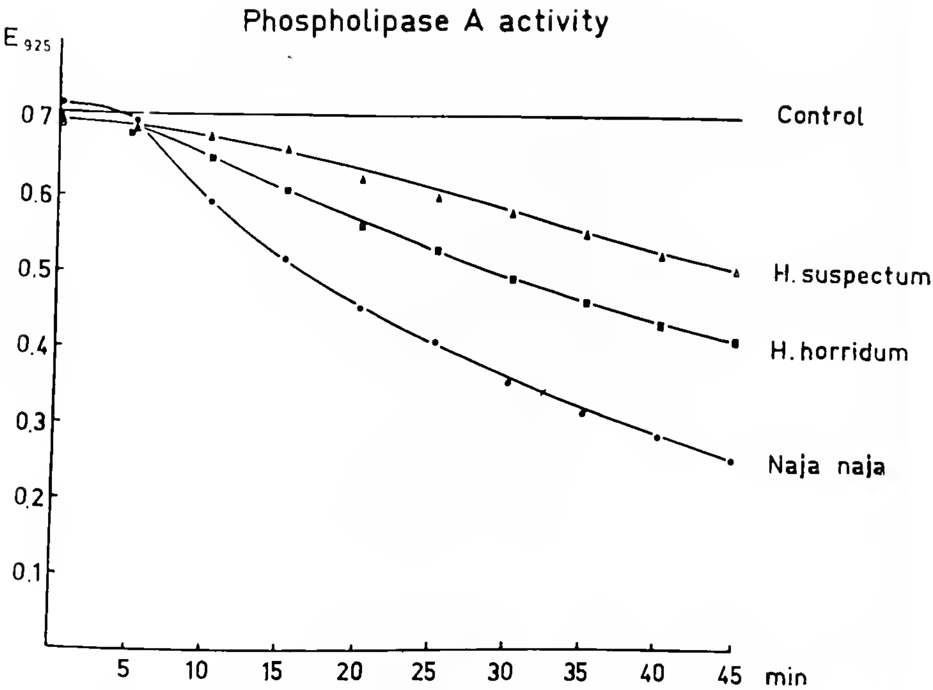

Fig. 2 — Phospholipase A activity of *Heloderma* venom.

Heloderma venom showed only a small proteolytic activity with a pH-optimum from 8.0-8.5, tested on casein (Fig. 3). As well as casein, denatured hemoglobin and gelatine were hydrolysed in small rates, likewise benzoyl-arginine-ethyl ester. The proteolytic activity was not affected by soybean trypsin inhibitor.

Heloderma venom incubated with bovine plasma globulin releases a substance which causes an atropin resistant contraction on isolated guinea pig ileum. This substance is heat stable and can be extracted from the incubate with hot alcohol and further precipitated with ether from the extract dissolved in acetic acid. Dialysing the alcohol extract against water, even after a relatively short time (3 hours), a part of the ileum contracting activity is found in the dialysate. By the addition of chymotrypsin to the incubate, the ileum contracting factor is rapidly inactivated. A contraction caused by venom-globulin incubate cannot be prevented by antihistaminica.

These facts indicate, that the substance which is released by venom from plasma globulin seems to be bradykinin or a substance like bradykinin. Kinin-release and casein hydrolysis showed the same pH-optimum (8.0-8.5). Paper electrophoresis showed the similarity of the venoms. At pH 6.5, 0.06 M phosphate buffer, seven fractions were observed (Fig. 4), at pH 8.0 only five. Beside the strongly toxic fraction which contains the protease and a part of the hyaluronidase, a further fraction, migrating to the cathode with a lesser toxicity, was separated. The other fractions were nontoxic and without enzymatic activity. *Heloderma* venom, as it is obtained, is a mixture of venom and saliva, so these inactive proteins could be originated from the cavity of the mouth.

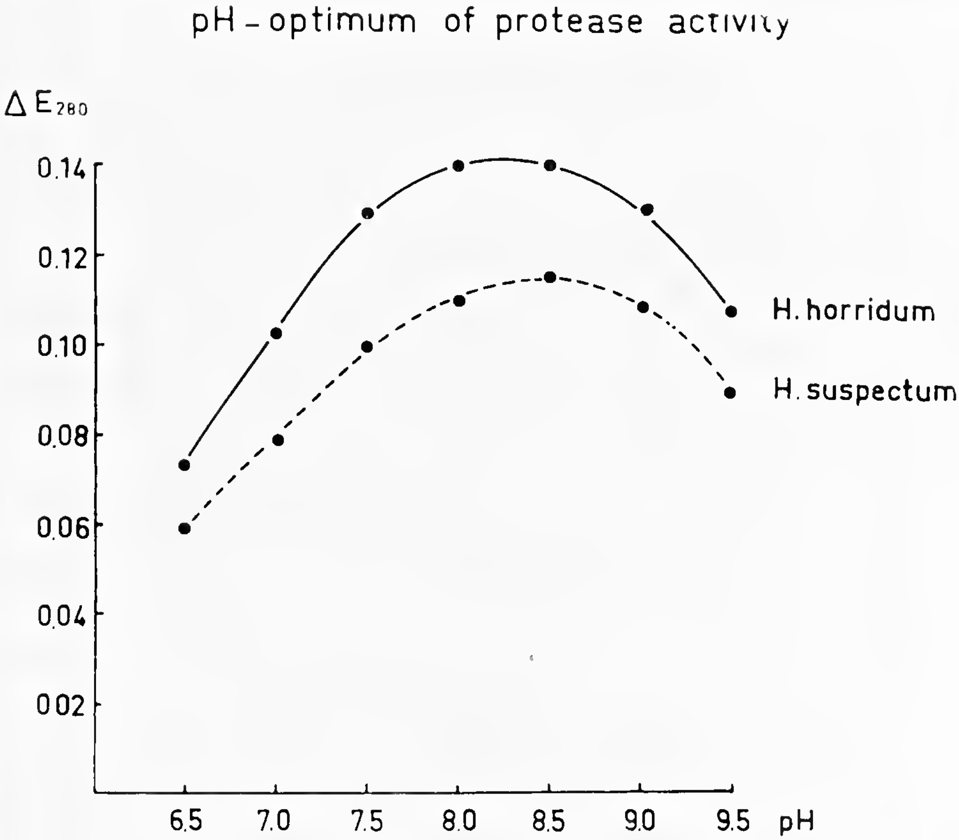

Fig. 3 — pH-optimum of the proteolytic activity of *Heloderma* venom, tested on casein splitting, incubation of 1% casein solution for 5 h, 37°C, with 250 μg venom each.

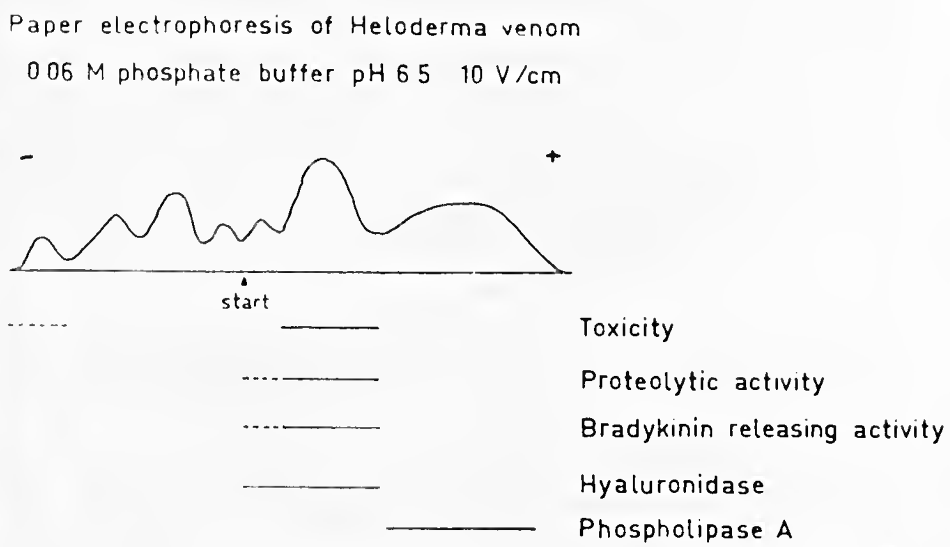

Fig. 4 — Paper electrophoresis of *Heloderma* venom, 0.06 M phosphate buffer pH 6.5, 10 V/cm.

Summary

The venom of *Heloderma suspectum* and *Heloderma horridum* possesses a toxicity comparable with that of cobra venom. It contains a very active hyaluronidase, a phospholipase A and a kinin-releasing enzyme with small proteolytic activity and a pH-optimum of 8.5. Electrophoretically both venoms are similar.

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33. INHIBITION OF ELECTRON TRANSPORT CHAIN BY PURIFIED PHOSPHOLIPASE A FROM *BOTHROPS NEUWIEDI* VENOM

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Introduction

It is known that snake venoms inhibit mitochondrial electron transfer systems (1-5). Badano and Stoppani (6, 7) studied the action of crude and heated venoms of several species of *Bothrops* on nonphosphorylating heart muscle extracts (Keilin and Hartree preparation) and found that the inhibitory power was proportional to the phospholipase A activity of those venoms. In this paper studies with purified phospholipase A (phosphatide acyl-hydrolase, E.C. 3.1.1.4) from *B. neuwiedi* venom are described.

Materials and methods

Enzyme preparations — Keilin & Hartree heart-muscle preparations were obtained from pig heart according to Slater (8). Protein concentration was measured with the biuret method (9).

Measurement of catalytic activity of heart-muscle preparation — These were carried out spectrophotometrically at 30° (6, 7, 10). Reaction mixtures (final vol. 3.0 ml) were made up as follows: a) $NADH_2$-oxidase: 0.25 mM $NADH_2$, 0.04 mM cytochrome *c*, 0.13 M phosphate buffer, pH 7.4; b) $NADH_2$-cytochrome *c* reductase: 1 mM KCN, 0.07 mM cytochrome *c* (oxidant) and other conditions as in (a); c) $NADH_2$-CoQo reductase: 0.13 mM Qo (oxidant); other conditions as in (b); d) Menadiol oxidase. Conditions as described by Colpa-Boonstra and Slater (11); e) $NADH_2$-dehydrogenase. Conditions as described by Minakami *et al.* (12); f) Succinate oxidase: 28 mM succinate, 0.02 mM cytochrome *c* and 0.13 M phosphate buffer (pH 7.4); g) Succinate dehydrogenase: conditions as described by Arrigoni and Singer (13); h) Cytochrome oxidase: conditions as described by Smith (14).

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Abbreviations: $NADH_2$, nicotinamide adenine dinucleotide, reduced form; Q_0, 2, 3-dimethoxy-3'-methyl-*p*-benzoquinone; EDTA, ethylenediaminetetra-acetate; DEAE-cellulose, diethylaminoethylcellulose; A, absorbancy; cyt. *c*, cytochrome *c*; K_3H_2, menadiol; PMS, phenazine methosulphate; Tris, tris (hydroxymethyl) aminomethane.

Measurement of phospholipase A activity — The following methods were used: a) The turbidimetric method of Marinetti (15). In those conditions one unit of enzymatic activity was defined as the amount of venom that, in 10 min produced a decrease of 0.010 in absorbancy at 925 mμ. *B. neuwiedi* venom presents a "lag" period before enzymatic activity is observed, and therefore the change in absorbancy at 925 mμ was considered after the end of the "lag" period. b) The method of Habermann and Neumann (16). The inhibition of coagulation of an eggyolk suspension incubated with venom is compared with a control sample incubated in the same conditions but without venom. With this method one unit of enzymatic activity was defined as the amount of venom that increases in one minute the coagulation time of the control. This method does not detect the "lag" period. c) The method of Magee and Thompson (17) was used with purified phospholipase fractions. Lecithin hydrolysis was followed by measuring the concentration of ester bonds by hydroxamate formation, according to Synder and Stephens (18). The hydrolysis required Ca^{++} ions as it has been established for phospholipase A obtained from other sources (19).

Protein concentration was measured by absorbancy at 280 mμ with the corresponding correction for nucleotide absorbancy.

RESULTS

Purification of phospholipase A — The method described by Saito and Hanahan (20) slightly modified (21) was applied to dried venom.

Table I shows the several steps of enzyme purification. In gel filtration (step 2), all material with the phospholipase A activity was excluded and an another fraction (about 16%) without enzymatic activity remained included in the column.

TABLE I — PURIFICATION OF PHOSPHOLIPASE A FROM VENOM OF *BOTHROPS NEUWIEDI*

Experimental conditions as in Fig. 2. Enzyme activity measured by the turbidimetric method (ref. 15).

Step.	Total activity (Units)	Total activity Yield %	Specific activity units / mg of protein	(a)
Crude venom	3234	100	54	(1.0)
1. Heat-treatment at pH 3.0	3168	97	115	(2.1)
2. Gel-filtration through Sephadex G-25	3798	117	168	(3.1)
3. Chromatography on DEAE-cellulose:				
F_1 (Tube No. 7-19)	380	12	73	(1.7)
F_2 (Tube No. 53-65)	2887	84	1750	(32.0)
F_3 (Tube No. 79-84)	491	15	614	(11.4)

(a) Increase in specific activity obtained at each step.

The elution diagram of DEAE cellulose chromatography (Fig. 1) shows three fractions (F_1, F_2, and F_3) having phospholipase A activity.

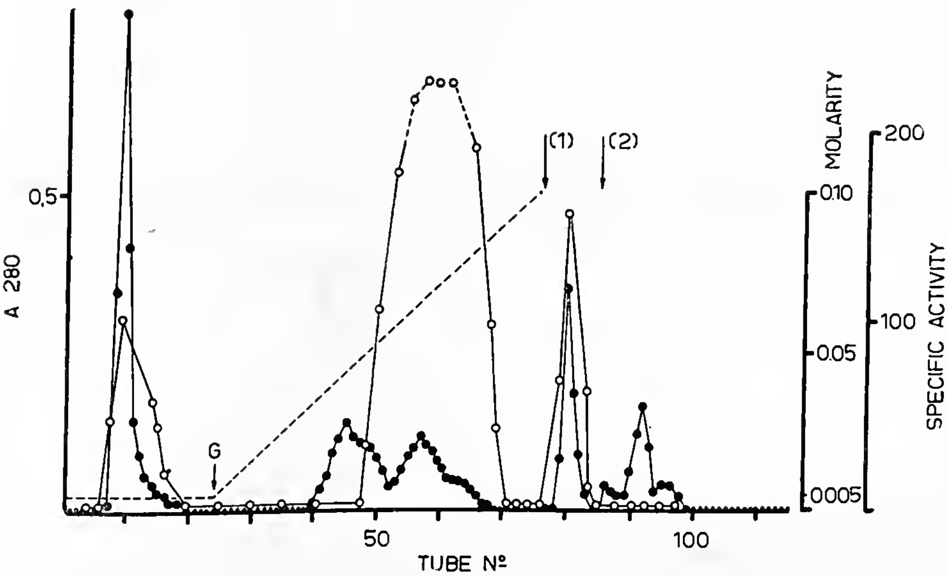

Fig. 1 — Chromatography of Step 2 fraction on DEAE-cellulose. 41 mg of protein were applied on top of a 21 × 1.2 cm DEAE-cellulose. Elution with potassium phosphate buffer (pH 7.4). The gradient was initiated at arrow G. Arrows (1) and (2) indicate the points at which 0.1 M potassium phosphate buffer pH 7.0, and 1.0 M potassium phosphate buffer, pH 7.0, were used as eluants. ● Absorbancy at 280 μm; O phospholipase specific activity (units/mg) measured by the turbidimetric method (15).

The phospholipase A activity in the crude venom samples presented an appreciable "lag" period (Fig. 2) that was not affected by the concentration of venom in the reaction mixture, by the addition of Ca^{++} ions or by increase of the temperature at which activity was measured. On the other hand, the active fraction obtained after Steps 2 and 3 of the purification procedure did not present any "lag" (Fig. 3). Similarly, autolysis of crude venom samples suspended in saline at 15°C produced a progressive disappearance of the "lag" period with simultaneous increase in total phospholipase activity (Fig. 4). The effect of autolysis could be observed only if venom proteases were active.

These facts suggest the presence in crude venoms of a low molecular weight peptide inhibitor of phospholipase A. The inhibitor would form a termostable complex with the enzyme at pH 4.5 but at pH 7.4 the complex would dissociate and then the components could be separated on Sephadex G-25. This interpretation is confirmed by the direct demonstration of an inhibitor of phospholipase A in the included fraction obtained from Sephadex G-25. In fact, in another series of experiments (21), it was found that when the included fraction was added to the active one, it provoked a "lag" with the same characteristics as that of the crude venom and simultaneously, the included fraction produced a strong inhibition of phospholipase activity. No inhibitory action was found in the included fraction obtained from samples of "autolyzed" venom (see above), nor in samples of the included fraction preincubated with trypsin.

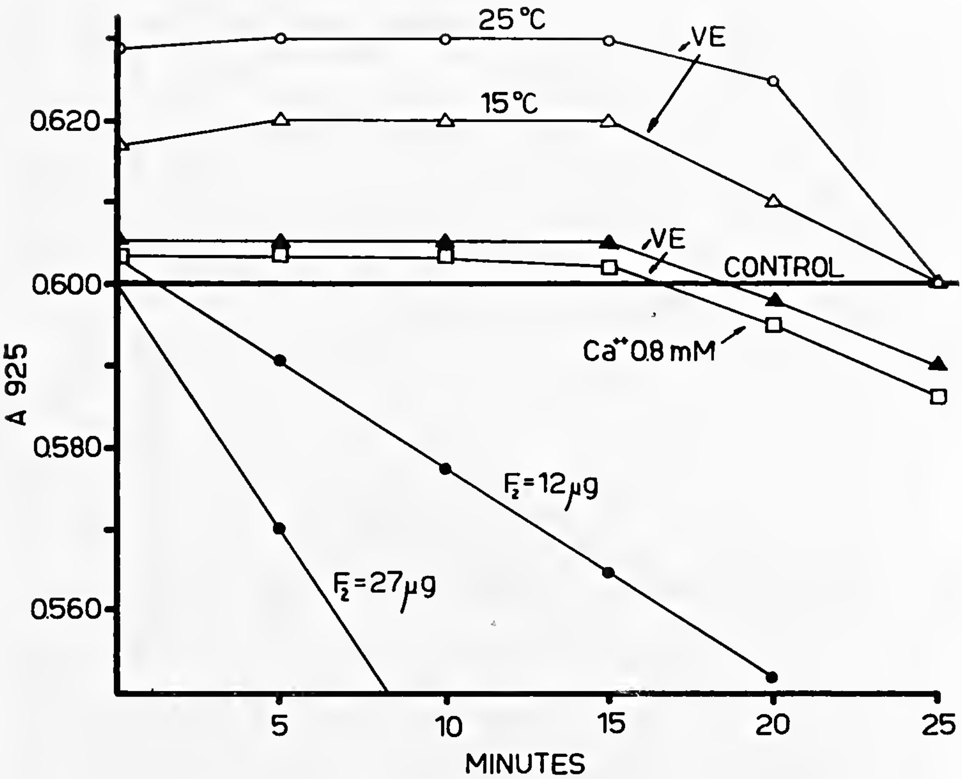

Fig. 2 — Effect of venom concentration, temperature and Ca^{2+} addition on the "lag" period. Enzyme activity measured turbidimetrically (15). The reaction mixtures were incubated at 25° with 88 µg (○) or 42 µg (▲, □) of crude venom, or at 15° with 88 µg of crude venom (△). VE: crude venom. The other curves (●) were obtained at 25° with the stated amount of F_2.

Fraction F_2 which appears homogeneous after electrophoresis on polyacrylamide gel, was tested as inhibitor of oxidizing heart muscle extracts.

According to Table II (Expts. *A* and *F*), treatment with phospholipase A produced a significant inhibition of the $NADH_2$-oxidase and succinate oxidase systems. The addition of cytochrome *c* (0.04 mM) lowered the inhibition of the $NADH_2$-oxidase system to 78%.

In order to establish the phospholipase-sensitive sites, the activity of the different components of electron transport chain was measured using adequate electron acceptors (or donors) and/or blocking cytochrome oxidase activity with KCN. Neither $NADH_2$-dehydrogenase (Expt. *E*) nor succinate dehydrogenase (Expt. *G*) were affected after phospholipase treatment. This result is very interesting because it has been demonstrated (9, 22) that digestion with phospholipase A produced the solubilization of $NADH_2$-dehydrogenase. However, it is possible that in the experiments described in Table II the concentration of enzyme used was not sufficient to produce liberation of a significant amount of $NADH_2$-dehydrogenase. It must be recalled that under similar experimental conditions Badano and Stoppani (7) found complete inhibition of succinate dehydrogenase after

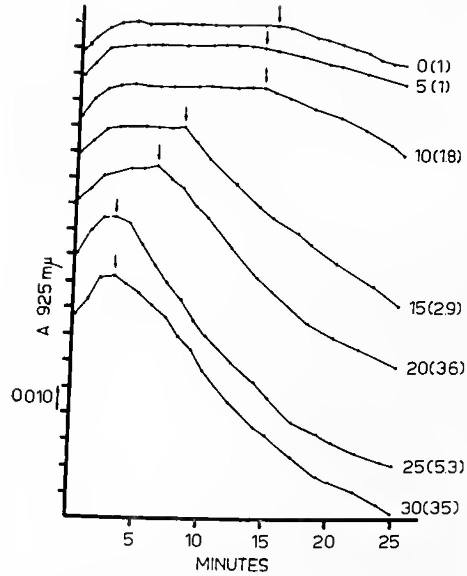

Fig. 3 — Effect of autolysis on the "lag" period. Samples containing 250 μg of crude venom were dissolved in 1 ml 0.9% (w/v) NaCl and incubated at 15° for the time (minutes) stated at the end of each plot. Phospholipase activity was measured by the turbidimetric method (15). The data in parentheses indicate the relative phospholipasic activity of the autolysed sample versus that of the original control sample.

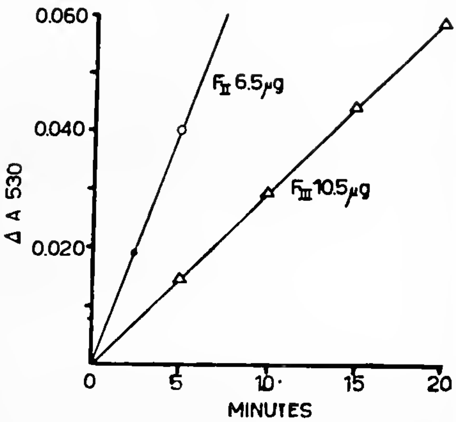

Fig. 4 — Rate of hydrolysis of lecithin by purified F_2 and F_3 measured the turbidimetric method (15). 10 mg sonicated lecithin in aqueous suspension was incubated with enzyme as stated. Reaction mixture: lecithin 5 mg/ml; 50 mM Tris-HCl buffer (pH 7.4), 1 mM EDTA and 5 mM $CaCl_2$. Final volume 2.0 ml.

treatment of Keilin-Hartree preparation with crude venom. Since succinate dehydrogenase is inhibited by treatment with proteolytic enzymes, they interpreted (7) the enzyme inhibition as due to the venom proteases. The present results support that interpretation.

Reduction of Qo (Exp. *C*) by the phospholipase treated preparation ($NADH_2$-Qo reductase activity) was partially inhibited but the magnitude of this inhibition was not sufficient to explain the inhibition of the whole system. On the other hand, menadiol oxidase (Expt. *D*) and $NADH_2$-cytochrome *c* reductase (Expt. *B*) activities were strongly inhibited, in agreement with the inhibition of the whole system. Finally, cytochrome oxidase activity (Exp. *H*) was partially inhibited.

In order to ascertain whether the inhibitions presented in Table II were due to alteration of the lipid constituents of the electron-transport particles, phospholipase-treated and control preparations were extracted according to Fleischer *et al.* (23) and analysed by thin-layer chromatography on Silicagel G (24). After revelation, phosphorus was determined in each spot according to Doizaki and Zieve (25). Such studies showed a decrease in the concentration of lecithin, phosphatidyl-ethanolamine and a third component (probably phosphatidic acids) and a correlative increase in the respective lysoderivatives by an amount that represents about 40% of the total phospholipid content (26).

TABLE II — EFFECT OF TREATMENT WITH PHOSPHOLIPASE A ON THE CATALYTIC PROPERTIES OF THE KEILIN-HARTREE HEART-MUSCLE PREPARATION

Expt.	Reaction	Control preparation (Sp. activities)*	Inhibition of activity by phospholipase A (%)	
			10 min incubation	80 min incubation
A	$NADH_2 \longrightarrow O_2$	0.674	57	85
B	$NADH_2 \longrightarrow$ cyt. c^{3+}	0.175	67	73
C	$NADH_2 \longrightarrow Q_0$	0.483	58	61
D	$K_5H_2 \longrightarrow O_2$	10.0	71	71
E	$NADH_2 \longrightarrow Fe(CN)_6^{3-}$	8.4	0	1
F	Suc. $\longrightarrow O_2$	0.539	41	48
G	Suc. $\longrightarrow$ PMS	2.5	−2	0
H	Cyt. $c^{3+} \longrightarrow O_2$	125.0	61	65

* *Specific activities:*

Expts. *A*, *B*, *C*, and *E*, μmole of $NADH_2$ oxidized/min/mg protein;

F and *G*, μmole of succinate oxidized/min/mg protein;

D and *H*, $k'/\min^{-1}$ (mg protein/ml)$^{-1}$ (k', constant of the first order equation).

Discussion

The role of phospholipids in electron transport has been studied in detail by Brierley *et al.* (27) and Fleischer *et al.* (23). It is well known that lipids represent 26% of mitochondrial weight; that phospholipids represent 90% of total lipids and that they are indispensable for electron transport. In fact, extraction with adequate solvents or enzymatic hydrolysis by phospholipases inhibits that transference.

The present study indicates that phospholipase A produces its inhibitory effect by acting on at least three points. 1) The segment Qo-cytochrome *c* is the most sensitive to phospholipase A attack. Elective inhibition of electron transport between quinone and cytochrome *c* seems to be a rather common property of phospholipases (28). 2) The fact that the quinone (Qo) reductase activity was affected, while $NADH_2$-dehydrogenase activity did not show any decrease, indicated that another site lies between the flavoprotein ($NADH_2$-dehydrogenase) and Qo. 3). The third point is near the cytochrome oxidase.

These conclusions which are in agreement with claims by other authors (2, 3, 5), show that the inactivation is in fact due to phospholipase A action, but it remains undecided whether it is caused by the hydrolysis of phospholipid constituents of the electron transport particle ("structural damage"), by the action of the products of hydrolysis on the electron carriers, or by both. When extracted submitochondrial lipids were treated with phospholipase A and the hy-

drolysate was added to the Keilin and Hartree preparation there was some inhibition of $NADH_2$-CoQo reductase and menadiol oxidase activities but in a lesser extent than after direct treatment of $NADH_2$-oxidase with phospholipase A. Succinate oxidase and cytochrome oxidase activities were not inhibited by the hydrolysed lipid extract. The difference between the "direct" and "undirect" inhibition of the electron transport system must be attributed to "structural damage" by phospholipase A (26).

Summary

Phospholipase A from *Bothrops neuwiedi* venom was purified by a procedure involving heat treatment at pH 3.0, gel-filtration through Sephadex G-25 and chromatography on DEAE-cellulose. Three active fractions were isolated (F_1, F_2 and F_3) with specific activities 1.7, 32 and 11 times, respectively, higher than that of the crude venom sample. F_2 and F_3 migrated as homogeneous proteins after electrophoresis on polyacrylamide gel. The crude venom contains an inhibitor of phospholipase A activity which is responsible of the initial "lag" period in the development of enzyme activity and can be separated by filtration through Sephadex G-25 since it remains in the included fraction. When this fraction was added to the active fractions it provoked a prolonged "lag" and a strong inhibition of activity. The inhibitory effect was nullified after treatment with trypsin or by autolysis of entire venom at 15°. The incubation of submitochondrial heart muscle particles (Keilin-Hartree preparation) with F_2 intensely inhibited $NADH_2$-oxidase, succinate and $NADH_2$-cytochrome *c* reductase activities. There was a less effective inhibition of menadiol oxidase. $NADH_2$-Qo reductase and cytochrome oxidase, but $NADH_2$-dehydrogenase and succinate dehydrogenase activities were not affected after treatment with phospholipase A.

Acknowledgments — This work was supported by grants of Consejo Nacional de Investigaciones Científicas y Técnicas and The Jane Coffin Childs Fund for Medical Research. Dr. A. Barrio kindly supplied *Bothrops neuwiedi* venom.

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34. PREPARATION AND PROPERTIES OF ^{131}I-LABELLED CROTOXIN

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The *in vivo* distribution of snake venoms is still very little known. Gennaro and Ramsey (1) applied the radioisotope trace technique to get some information concerning this important aspect of venom pharmacology. They labelled the cottonmouth moccasin (*Agkistrodon piscivorus*) venom with iodine-131 and determined the radioactivity of various tissues from mice injected with this material. However, the utilization of labelled crude venom does not seem to be well suitable for this purpose. Snake venoms are complex mixtures of proteins whose molecular weight, composition in amino acids, toxicity and pharmacological actions widely differ. Suppose, for instance, the specific activities of the various components of the labelled venom under investigation are not the same. Then the radioactivity of the tissues from the animals injected with it will not give a real picture of its distribution. Moreover, the correct distribution of the more toxic components, what is really the important thing to be known, can not be clarified by using labelled crude venoms due to presence of inactive and little toxic labelled proteins in them. Therefore, it seems better to label venom components, instead of whole venoms.

A good knowledge of the pharmacology and role played in the physiopathology of envenomation of the component to be labelled is highly desirable. The labelled component may be used, in distribution studies, either alone or incorporated in the crude venom. Experiments with the last mentioned material seem to be necessary as some venom components may alter the rate of absorption of other venom components and the biological barriers of the organism, thus affecting their distribution.

An investigation on distribution, points of fixation in tissues at cellular and molecular levels, rate and pathways of absorption and excretion of snake venoms has been planned according to the criteria outlined. The venom of the Southamerican rattlesnake (*Crotalus durissus terrificus*) was selected for this study and the labelling of crystalline crotoxin, its main toxin, with radioiodine, tried. The method of iodination used to label crotoxin as well as the physicochemical and biological characteristics of the radiocrotoxin obtained are reported in this paper.

This research was supported by a grant from Fundação de Amparo à Pesquisa do Estado de São Paulo.

Material and methods

Crotoxin — Crystalline crotoxin was prepared according to the methods developed by Slotta and Fraenkel-Conrat (2). Solutions for iodination were made at the time of the experiments by dissolving 30 mg of crotoxin in 10 ml of saline. The pH of the solutions, originally between 4.5 and 5.0 was adjusted to 8.0.

Counting — A NaI (Tl) 3″ × 3″ well-type scintillation counter was used with the pulse-height analyzer set at the ^{131}I keV photopeak. Counts were accumulated during a time sufficient to achieve a statistical error not greater than 3%.

[^{131}I] iodide — Sodium [^{131}I] iodide, carrier-free, was supplied by the Radiochemistry Division of the Atomic Energy Institute, São Paulo, in a strongly alcaline solution (pH 12.0). The radioiodine used was free of reducing agents.

Iodination — McFarlane's (3) method was used. Pre-oxidation of the protein as suggested by McFarlane was employed in two preliminary experiments. It was carried out with a solution of iodine-iodide at pH 4.5 followed by filtration of the mixture through a IR-4B Amberlite column in order to remove the excess of iodine. The pH of the eluates was adjusted to 8.0 or 9.0. No pre-oxidation was used in the 12 subsequent experiments.

After iodination, unbound radioiodine was usually remove from the mixture by an ion exchange resin (Amberlite IR-4B). In one of the experiments, part of the mixture was submitted, for this purpose, to dialysis against running water for 24 hours at room temperature.

The adopted procedure to label crotoxin with iodine-131 was as follows. The pH of a sodium [^{131}I] iodide solution with total activity from 3 to 8mCi was adjusted to pH 8.0 or 9.0 with hydrochloric acid and buffered with alcaline glycin (pH 8.5). Then, 0.7 ml of a ^{127}I Cl solution containing 2.94 mg of iodine were added in order to obtain the ^{131}I Cl (Iodine-131 monochloride) by isotopic exchange. The crotoxin solution was jet sprayed over this "iodination mixture", and after strong agitation and 2 to 4 minutes of rest, passed through the anionic resin Amberlite IR-4B. The total activity was then determined.

Physicochemical assays — The radiochemical purity of the iodinated material was investigated by thin layer chromatography (silica gel G, 250 μ thick). a mixture of acetone, n-butanol, concentrated amonia and water (65:20:10:5) being used as solvent. The running time was 25 minutes. Crotoxin which remained at the origin ($Rf = 0$) was developed by nihidrin, and iodine, that migrated with the solvent ($Rf = 0.7$), by lead acetate. After drying, the plates were divided in three parts and counted. Unbound iodine in the ^{131}I-labelled crotoxin solution was also estimated by paper electrophoresis (Whatman n.º 1). It was performed with barbital-sodium barbital buffer $pH = 8.6$, $\mu = 0.05$ and 2 mA per strip for 50 minutes. The labelled crotoxin did not migrate ($Rf = 0$).

Iodination of the crotoxin molecules was verified by the trichloroacetic acid test. The protein was precipitate by adding the acid to the mixture, the precipitate obtained being washed several times and its radioactivity finally determined.

Labelled crotoxin was compared with the original crotoxin by means of paper electrophoresis. The buffer, ionic strength and pH were as referred. Running time of 4 and 18 hours were used. The electrophoretic strips containing the radiocrotoxin were also analyzed in the "Vanguard radioscanner".

Protein determination — Protein (crotoxin) concentration in the Amberlite eluated was estimated by determining its nitrogen content. The micro-Kjeldahl and the ultra-violet (260 mμ and 280 mμ) differential spectrophotometry methods were used. The nitrogen content of the original crotoxin was also determined.

Biological assays — The toxicity of different batches of labelled crotoxin was compared with that of the original ones by determining their median lethal doses for piedgeons and mice. Four dose levels in geometrical progression (common factor 1.5) and six or exceptionally ten animals per dose were used. The animals were observed for a period of 24 hours. The median lethal doses and their 95 of 100 confidence intervals were calculated by Weil procedure (4).

The method of Cesari and Boquet (5) was employed for evaluating the haemolytic (phospholipasic) activity of the labelled and unlabelled crotoxin preparations. Inactivated sera and blood cells from dogs were used in these assays.

Four conscious dogs were intravenously injected with 0.25 mg/Kg of the radioiodinated crotoxin. Three of them were prepared as described in a previous paper (6), for recording contractions of the tibialis anterior muscle and arterial blood pressure, after being anaesthetized with sodium pentobarbital (20 mg/Kg. i.v.). Two dogs were prepared 24 and one 48 hours after the injection of the labelled crotoxin. Stimulation of the peroneal nerve was carried out with supramaximal square wave pulses of 0.2 msec and 0.1 c/sec. When stimuli up to 100 v did not elicit muscle responses, it was assumed that neuromuscular transmission was completely blocked. Direct muscle stimulation was done with shocks of 50-100 v, 2 msec and 0.1 c/sec.

One of the four dogs was sacrificed nine days after the administration of the radioiodinated crotoxin. The kidneys of this dog and those from the animal which was used for studying neuromuscular transmission 48 hours after radioiodinated crotoxin administration were removed for histopathological examination. The urines from these dogs were examined for albumin and haemoglobin.

The action of the labelled crotoxin was compared with that of the original one on the isolated guinea-pig ileum. The intestine was suspended in an organ bath with Tyrode solution at 37°C. A frontal inscription isotonic lever was employed. The preparation was oxygenated with air.

Results

A good labelling efficiency did not occur when pre-oxidation was performed. In the remaining experiments. a yield of 35 to 40 per cent of the initial radioactivity was obtained. The specific activity of the radioiodinated crotoxin was in this case always greater than 120 μCi/mg.

The evaluation of unbound iodine by chromatographic and electrophoretic methods gave identical results. There was from 2 to 6 per cent of free ^{131}I in the different preparations. On the other hand, the trichloroacetic acid test

showed that from 94 to 98 per cent of the total radioactivity were present in the precipitates.

No differences were detected in the electrophoretic behaviour of crotoxin and radiocrotoxin (Fig. 1). Analysis of the electrophoretic strips of labelled crotoxin in the radioscanner revealed the presence of two peaks (Fig. 1)..

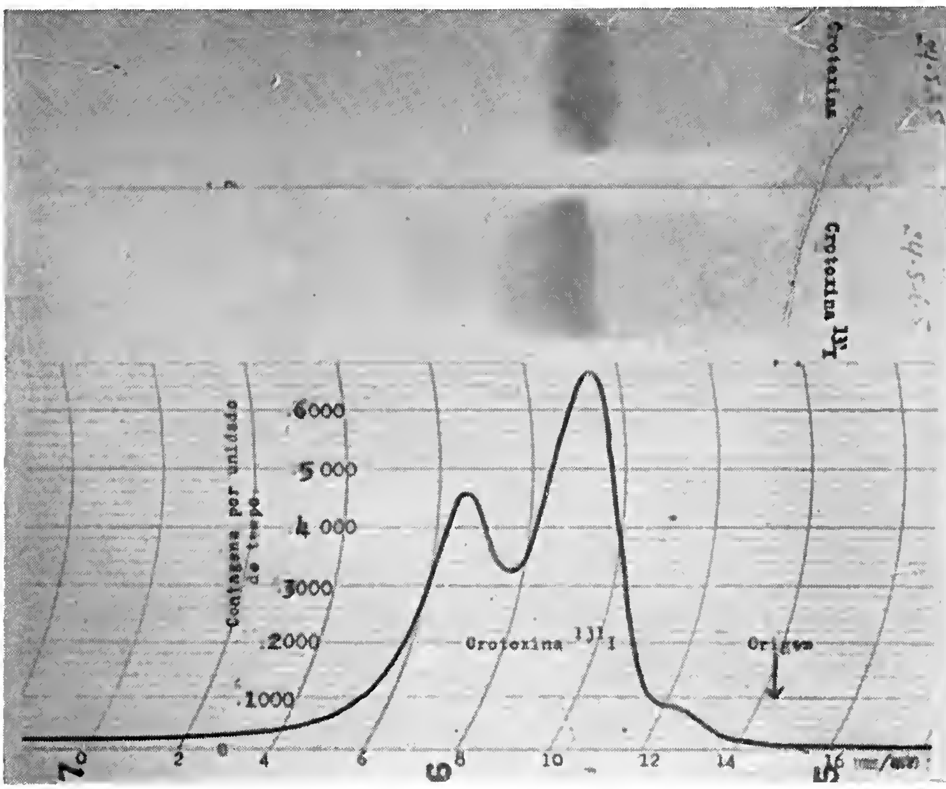

Fig. 1 — Top — Crotoxin and radiocrotoxin electrophoresis (pH 8.6, ionic strenght 0.05, 2 mA, veronal-sodium veronal buffer) strips. Bottom — Radioscanner of the labelled crotoxin.

The toxicity of radioiodinated crotoxin and that of crotoxin for pidgeons and for mice did not differ significantly. The median lethal dose, for instance, of a batch of crotoxin for pidgeons was 3.15 (1.62 to 4.18) mcg per Kg; after iodination, its LD_{50} was found to be 3.44 (3.40 to 3.45) mcg per Kg.

Table I and figure 1 show the median lethal doses of different batches of crotoxin and radioiodinated crotoxin for mice. The symptoms presented by the animals injected with crotoxin and radiocrotoxin were the same.

The haemolytic activity of the labelled crotoxin preparation which were passed through the anionic resin resulted somewhat lower than that of the original crotoxin. However, when the free iodine was removed by dialysis, the haemolytic activity of both preparations was the same (Table II).

TABLE I — TOXICITY FOR MICE (18 TO 21 g) OF SOME LABELLED AND ORIGINAL CROTOXIN PREPARATIONS

Doses in micro-grams	Results (deaths in 24 hours)				
	Experiment I (07/08/65)		Experiment II (18/09/65)		Experiment III (16/11/65)
	Crotoxin	Radiocrotoxin	Crotoxin	Radiocrotoxin	Radiocrotoxin
0.66	—	—	0/6	0/6	0/6
1.00	0/6	0/6	1/6	2/6	2/6
1.50	0/6	0/6	4/6	5/6	5/6
2.25	5/6	5/6	6/6	6/6	6/6
3.37	6/6	6/6	—	—	—

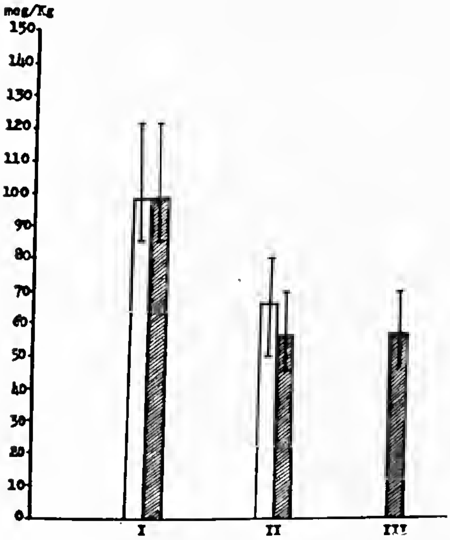

Fig. 2 — Mean lethal doses (mcg/Kg) and their 95 of 100 confidence intervals of the same preparations referred to in Table I.

Vomits, defaecation, long lasting flacid paralysis, albuminuria and haemoglobinuria were the symptoms presented by the dogs injected with the radioiodinated crotoxin. They were identical to those showed by dogs injected with crotoxin (7).

Partial or complete neuromuscular blockade (Fig. 3) was demonstrated in the dogs injected with radiocrotoxin. Therefore, neuromuscular action was identical to that of crotoxin (6).

The renal lesions found in the dogs injected with crotoxin (8) were also observed in the kidneys of the dogs intoxicated by ^{131}I-crotoxin.

The labelled crotoxin contracted the isolated guinea-pig ileus as crotoxin does (9). There was cross dissensitization between crotoxin and radioiodinated crotoxin.

TABLE II — *IN VITRO* HAEMOLYTIC ACTIVITIES OF CROTOXIN AND RADIOIODINATED CROTOXIN

Doses in micrograms	Crotoxin	^{131}I Crotoxin	^{131}I Crotoxin *
10	+++	+++	+++
5	++	+	++
2	+	0	+

* Dialysed radiocrotoxin.

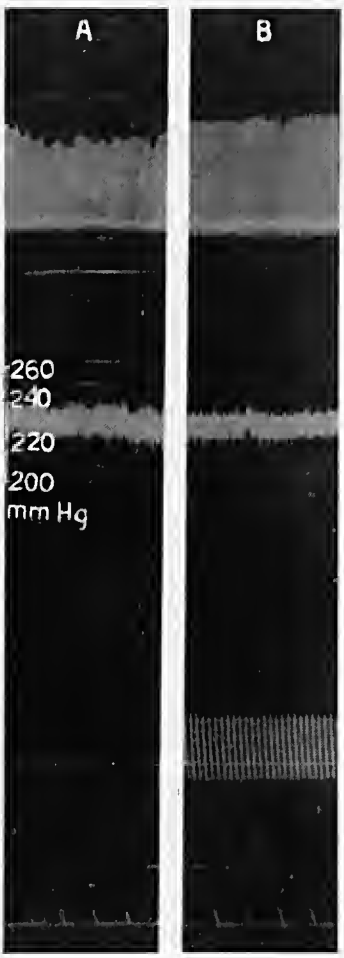

Fig. 3 — Records of the respiratory movements, blood pressure, and of the tibialis anterior muscle contractions of a dog (18 Kg, pentobarbital anaestesia), injected 22 hours before with 250 mcg/Kg of radiocrotoxin. A — Stimulation of peroneal nerve with square wave pulses of 0.2 msec., 0.1 c/sec and 100 v. B — Direct mucle stimulation with pulse of 2 msec, 0.1 c/sec and 100 v.

Discussion

The results provided by the trichloroacetic acid tests and by the analysis of the electrophoretic strips in the radioscanner showed that there was a real iodination of crotoxin, a protein or protein complex with a high aromatic amino

acid content (9). On the other hand, the specific activity obtained by the method of iodination used is sufficient for crotoxin distribution studies.

Iodination did not induced important physicochemical modifications in the crotoxin molecule. This can be inferred from the results of the biochemical and biological assays. The smaller haemolytic activity of the radiocrotoxin was not due to iodination. It could be traced to the passage through the anionic resin: A radioiodinated preparation submitted to dialysis instead of being passed through the resin, presented the same haemolytic activity as the original crotoxin.

Crystalline crotoxin behaves as a homogeneous protein with a molecular weight of the order of 30.000 in sedimentation and diffusion experiments carried out with the ultracentrifuge (10). It behaves also as a uniform protein when examined by Cohn method (2). Signs of nonuniqueness of crotoxin have not been observed by mean of electrophoresis either (11). However, in the present investigation it was shown by the analysis of the electrophoretic strips of radioiodinated crotoxin in the radioscanner the presence of two peaks of maxinal radioactivity. This seems to demonstrate that electrophoresis can start a separation of crotoxin in two components, a fact not revealed by the common procedures. It is relevant to remember that. according to Neuman and Habermann (12, 13) crotoxin is made up of two proteins: A phospholipase A of low toxicity and a toxin of high toxicity ("crotactin") which are probably linked by ionic bounds.

Summary

McFarlane's method of iodination was used for labelling crystalline crotoxin. the main toxin of the South American rattlesnake venom. with radioiodine. Yields of 35-40 per cent of the original radioactivity were obtained. Free iodine was removed by means of filtration through an anionic resin (IR-4B Amberlite) column. Radioiodinated crotoxin with specific activities greater than 120 μCi per gm was obtained.

Radioiodination of crotoxin was demonstrated by the trichloroacetic acid test and by submitting the electrophoretic strips of the iodinated preparations to analysis in the radioscanner.

Biochemical and biological assays of ^{131}I-labelled crotoxin revealed that the iodination did not induced important physicochemical modifications in the crotoxin molecule.

In view of the results of the present investigation. the authors suggest that the ^{131}I-labelled crotoxin is a convenient tool for the investigation of the distribution and rates of absorption and excretion of crotoxin as well as its points of fixation in tissues at cellular and molecular levels.

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35. ACTION DU VENIN DE *NAJA NIGRICOLLIS* SUR LA COAGULATION SANGUINE

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Parmi les ELAPIDAE africains, ceux de l'espèce *Naja* sécrètent un venin dont l'activité anticoagulante a été observée depuis fort longtemps (1).

Le mécanisme par lequel ces venins empêchent la coagulation du sang de se produire demeure mal expliqué. Le venin de *Naja nigricollis*, par exemple, ne présente aucun pouvoir anti-thrombique puisqu'il ne s'oppose ni à la coagulation du plasma, ni à celle du fibrinogène par la thrombine. L'effet inhibiteur se manifeste par contre au cours de la coagulation du sang total, du plasma recueilli sur oxalates et secondairement recalcifié, ou de la détermination du taux de prothrombine par la méthode de Quick. L'expérience montre également que l'incubation du venin de *Naja nigricollis* avec la thromboplastine tissulaire amène la destruction partielle de celle-ci. Malheureusement, dans des tests de coagulation plus complexes, la nature de l'effet anticoagulant n'apparaît pas clairement (2); à peu près toutes les hypothèses ont été envisagées concernant la destruction de tel ou tel facteur de coagulation et aucune certitude ne peut être acquise.

Substituant au venin brut de *Naja nigricollis* une fraction très anticoagulante (fraction III), obtenue par filtration du venin sur Sephadex G_{100} (3, 4), nous avons cherché à determiner la nature du facteur responsable de l'activité anticoagulante.

Les expériences de diffusion-précipitation (3, 4) montrent en effet la présence dans la fraction III de quatre antigènes.

L'un de ces antigènes est commun avec la fraction I. Un autre antigène a pu être identifié à la phospholipase. Le fait que la fraction I, soumise à un chauffage très bref à 96°, présente un pouvoir anticoagulant, suggère que le facteur anticoagulant est cet antigène commun aux fractions III et I. Mais sa nature demeure inconnue. Nous ignorons s'il s'agit d'une enzyme ou d'un inhibiteur. Aucune activité protéolytique ou estérasique n'a pu lui être imputée: la caséine, l'hémoglobine, la sérum-albumine ne sont pas hydrolysées, les esters synthétiques (TAME, BAME) ne sont pas scindés.

Si nous admettons que le facteur anticoagulant principal du venin de *Naja nigricollis* est cet antigène, devons-nous pour autant suivre Kruse et Dam (5) qui, comme Fleckenstein et Fettig (6), dénient toute activité anticoagulante à la phospholipase? Si l'on ajoute de très grandes quantités de phospholipides (Inosithin) à un plasma soumis à l'action de la fraction III du venin, on parvient à réduire considérablement l'effet anticoagulant observé. Il s'agit peut-être néanmoins d'un effet non spécifique et cette expérience ne prouve pas que la phospholipase dégrade les phospholipides nécessaires à la coagulation.

Outre cette activité anticoagulante difficile à préciser, le venin des *Naja* africains est capable de coaguler le sang de cheval. Boquet et Izard (7) observèrent en effet qu'à fortes doses, ce venin était susceptible, non plus de retarder, mais d'accélérer la coagulation.

L'addition de quantités croissantes de venin à du plasma humain exerce d'abord un effet anticoagulante qui augmente avec la dose de venin, puis un effet coagulant très intense.

La séparation du venin en différentes fractions (3, 4) permet de retrouver le facteur coagulant dans l'une d'elles (fraction I). La présence de calcium favorise l'activité de ce facteur, mais n'est pas indispensable. L'hypothèse de l'existence d'une enzyme de type thrombinique semble à exclure car la fraction I est totalement dépourvue d'action sur le fibrinogène pur. La possibilité d'une activation directe du facteur X par un mécanisme analogue à celui qui a été décrit dans le cas du venin de *Vipera russeli* (9, 10) ne nous a pas semblé devoir être retenue, car des plasmas de malades congénitalement privés de facteur X sont coagulés aussi vite que des plasmas normaux ou dépourvus des facteurs IX ou VII. Par contre, des plasmas adsorbés sur sulfate de baryum ou sur phosphate tricalcique, dépourvus des facteurs IX, VII et X ainsi que de la prothrombine vraie ne peuvent être coagulés. La présence de prothrombine apparaît donc comme indispensable à l'action du venin qui s'est révélé susceptible de coaguler seul une préparation contenant du fibrinogène pur et de la prothrombine purifiée. *

Certains auteurs actuels, parmi lesquels Magnusson (11), estiment que l'activation directe de la prothrombine en thrombine résulte d'une protéolyse. Or la fraction I du venin de *Naja nigricollis* (fraction qui comporte plusieurs constituants) ne présente aucune activité protéolytique sur l'hémoglobine, la caséine, la sérum-albumine et les acides aminés estérifiés. La nature de ce constituant du venin pose de ce fait un problème particulièrement complexe. Il s'agit d'un constituant par ailleurs très sensible à la chaleur: il disparaît après quelques secondes de chauffage à 96°. La mise en évidence d'un facteur accélérant la coagulation du sang dans le venin de *Naja nigricollis* nous a incité à rechercher une substance analogue dans les venins des autres *Naja* africains.

Une expérimentation récente a pu nous montrer que le venin de *Naja haje* possède, comme celui de *Naja nigricollis*, un facteur coagulant.

Il ressort de ce court exposé que l'étude de l'activité des venins d'ELAPIDAE africains sur la coagulation apporte des faits expérimentaux utiles à notre connaissance de la coagulation sanguine comme à celle du problème de la filiation de ces serpents au cours de l'évolution. Dans cet ordre de faits, il est intéressant de noter qu'à la différence des *Naja* vivant en Asie, les ELAPIDAE australiens des espéces *Notechis* et *Pseudechis* (12, 13, 14) présentent un très net pouvoir coagulant. On peut se demander quelle relation existe entre ce pouvoir coagulant et celui des ELAPIDAE africains que nous venons d'exposer.

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* Préparée par le Docteur J.-P. SOULIER.

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36. LES VENINS DE SERPENTS, SOURCE D'ENZYMES ANTICANCÉREUX

I. ASPECTS BIOCHIMIQUES FONDAMENTAUX DU PROBLÈME

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Introduction

Nous nous proposons d'exposer ici les résultats d'une recherche de trois années faite au Laboratoire de Biochimie de l'Institut Technique Supérieur de Chimie Meuriee de la Province de Brabant et au Laboratoire de Pharmacodynamie de l'Université Libre de Bruxelles.

Le but de cette recherche était de vérifier si les venins de serpents ont ou non une activité anticancéreuse et de tenter de situer cette action dans l'une ou l'autre des fractions enzymatiques du venin.

Un exposé exhaustif des bases théoriques et des hypothèses fondamentales de cette recherche a été fait dans un article publié cette année par L. Gillo (1). Ce problème n'était pas nouveau. La plupart des travaux expérimentaux sur les tumeurs d'origine animale et en clinique humaine ont été publiés entre les années 1930 et 1940. Les résultats qui en découlèrent furent souvent positifs mais aussi inconstants. Quatre groupes de savants dont Calmette, Saenz et Costil (2) attribuèrent au venin de Cobra une action antitumorale alors que cinq autres lui déniaient toute action: Essex et Priestley (3), Morelli (4), Morelli et Focosi (5), Julius (6), Vannfalt (7), Rosenbohm (8). La recherche d'une activité antalgique éventuelle donna environ 50% de résultats positifs (1).

Devant l'inconstance des résultats, la thérapeutique du cancer par les venins animaux fut abandonnée vers 1950.

A la lumière des connaissances actuelles sur la composition enzymatique des venins, nous pensons que cet échec doit être attribué à une erreur de conception faite par les auteurs. En effet, leur hypothèse était que l'action anticancéreuse était due aux composants nécrosants, aux agents cytolytiques, aux neurotoxines du venin et donc intimement liée à leur toxicité. Ainsi Calmette (2), puis Chopra et Chowhan (9) et Rousseau (10) ont attribué l'activité anticancéreuse aux lysolécithinases. Yiengar (11) et d'autres y voyaient l'action des protéases.

La plupart des chercheurs et cliniciens de l'école française, Grasset et des Ligneris (12), pensaient que cette action était liée aux toxines et titraient leurs préparations en unités-souris, unités basées directement sur le degré de toxicité. Il est compréhensible que, partant de telles hipóthèses et devant la nécessité d'utiliser des doses élevées pour atteindre le seuil d'activité, on ait abouti à l'intoxication de l'animal d'expérience ou du patient.

La croissance et la multiplication des cellules normales et cancéreuses ont été étudiées par les voies de la génétique biochimique et de l'enzymologie. L'étude du processus de croissance d'une cellule, de la synthèse des protéines cytoplasmiques sous le contrôle des acides nucléiques, des enzymes intracellulaires et des processus énergétiques rend très probable la possibilité d'une intervention des venins de serpents sur les processus cancéreux, par le moyen de leurs nucléases, leurs phosphatases, leurs ATPases, leurs inhibiteurs de déshydrogénases et bien d'autres enzymes non toxiques.

Contrairement à la conviction des premiers chercheurs, l'action antitumorale ou antalgique éventuelle est due à des enzymes non toxiques. Les anciens chercheurs ont fait une erreur de conception en attribuant cette action aux toxines. Par conséquent, dans les recherches expérimentales futures, les toxines et les lysolécithinases devront être écartées du complexe venimeux pour laisser apparaître l'action des enzymes non toxiques responsables de l'effet antitumoral ou antalgique.

Resultats et discussion

Dans nos expériences, nous avons choisi le même venin que Calmette, le venin de Cobra, que a l'avantage de n'être ni protéolytique ni nécrosant. Nous avons étudié sur des milliers d'animaux l'action antitumorale de doses de $\frac{1}{4}$ à 1 gamma de venin de *Naja naja*, *Naja naja atra* et *Naja melanoleuca*, sur 9 types de tumeurs spontanées et greffées. Les méthodes utilisées à cet effet sont décrites dans la communication du Docteur Wirtheimer.

Nous avons pu démontrer que le venin total de *Naja naja atra* est tout aussi actif sur le carcinome d'Ehrlich que l'Actinomycine D, médicament chimiothérapique classique, inhibiteur du RNA messager. Ce venin total est moins toxique que l'Actinomycine D. Pour savoir si cette activité antitumorale était due aux toxines ou aux non toxines, nous avons approché ce problème par deux voies, la première consistant à apporter la preuve que les toxines ne sont pas actives, la seconde en démontrant que les enzymes, peu ou non toxiques, ont une activité.

Nous avons appliqué le test "in vitro" sur le carcinome d'Ehrlich à la cobratoxine par cinq fois recristallisée *. Cette cobratoxine est estrêmement toxique: sa LD_{50} est de 60 γ/Kg. Elle s'est révélée totalement inactive même aux doses sublétales de 75 γ/Kg qui entrainent rapidement la morte de plus de la moitié des souris.

La même expérience fut faite avec la lysolécithinase extraite par nous de notre venin de Cobra par chromatographie sur TEAE-cellulose. En présence et en absence de lécithine, cet enzyme réputé toxique et admis par beaucoup d'auteurs comme le principal agent anticancéreux n'a aucune action inhibitrice sur le carcinome d'Ehrlich même aux doses sublétales. Il est important d'ajouter qu'à toutes doses l'examen microscopique démontre que la lécithinase n'a aucune action destructrice sur cette cellule contrairement à ce qu'on a toujours pensé.

En conséquence nous pouvons conclure que ni la cobratoxine, ni la lysolécithinase ne sont directement actives sur le cancer d'Ehrlich.

Pour vérifier si certaines fractions, peu ou non toxiques, du venin de *N. n. atra* possédaient une activité anticancéreuse, le venin fut soumis aux méthodes

* Cette cobratoxine nous a été aimablement envoyee par le Prof. Yang du Kaohsuing Medical College — Kaohsuing — Formose.

classiques de fractionnement. Les pics obtenus furent caractérisés suivant leur toxicité, leur composition enzymatique et de plus leur pouvoir d'arrêt de la croissance et de la propagation du carcinome d'Ehrlich.

Certaines fractions sont très actives et peu toxiques, quelquefois vingt cinq fois moins nocives que le venin total dont elles étaient issues.

Le venin agirait à l'extérieur de la cellule en dégradant par voie enzymatique le facteur de propagation (13).

Resume

Il a été démontré que le venin de *Naja naja atra* a une action inhibitrice sur le carcinome d'Ehrlich.

Contrairement aux hypothèses des précédents chercheurs, cette action n'est pas liée aux fractions toxiques.

Il a été démontré que les fractions le plus toxiques du venin, soit la cobratoxine et la lysolécithinase sont dépourvues d'activité anticancéreuse. D'autre part, des fractions qui sont jusque vingt cinq fois moins toxiques que le venin total se sont révélées très actives.

Reconnaissance — Nous tenons à exprimer ici toute notre reconnaissance à la Province de Brabant. à la F. W. Breth Foundation de New York et à Monsieur Mattens de Bruxelles dont la confiance et le soutien matériel nous ont permis d'effectuer cette recherche.

Summary

It was demonstrated that *Naja naja atra* venom has an inhibitory action on the Ehrlich's carcinoma.

Contrary to previously supported theories, this action is not due to the toxic fractions: cobratoxin and lysolecithinase, the most toxic components of the venom, show any anticancerous action. Fractions 25 times less toxic than initial venom appearead to be very active.

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37. LES VENINS DE SERPENTS, SOURCE D'ENZYMES ANTICANCÉREUX

II. ÉTUDE EXPÉRIMENTALE

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Matériel et techniques

Nos recherches ont porté sur des souris porteuses de 10 espèces differentes de tumeurs spontanées ou greffées.Notre choix fut guidé par la facilité d'élevage de ces animaux et la possibilité de disposer facilement de tumeurs variées:

TUMEURS SPONTANÉES (1)

Ces tumeurs de nature mammaire apparaissent spontanément entre le 5e et le 9e mois chez les souris de lignée C_3H ou R III dans des conditions particulières. Ces souris nous ont été fournies par le Centre Anticancéreux de Villejuif et par le Professeur Rudali de l'Institut Curie de Paris.

Les tumeurs se développent en quelques mois d'une manière irrégulière tout comme les tumeurs humaines et peuvent atteindre un volume énorme correspondant au tiers du poids de l'animal. Les métastases externes sont fréquentes et on observe quelquefois un envahissement des organes internes. Nous n'avons jamais observé de regression spontanée de ces tumeurs.

La nature histologique de cette tumeur est celle d'un adénocarcinome mammaire. Elle est fortement vascularisée, de nature kystique tubulaire pleine ou mixte. Des zones de nécrose ne s'observent que dans les très grosses tumeurs.

TUMEURS GREFFÉES

Deux variétés de tumeurs greffées d'origine mammaire nous ont été fournies par les Professeurs Mühlbock d'Amsterdam et Neukomm de Lausanne (2, 3). Elles sont greffées sur des souris C_3H et Swiss et leur évolution est plus rapide et plus régulière que celle des tumeurs spontanées.

Contrairement aux tumeurs spontanées dont la localisation se fait au hasard au niveau des nombreuses glandes mammaires, on peut choisir une place d'implantation favorable, notamment dans la paroi abdominale latérale.

TUMEUR KOPROWSKA

Cette tumeur, greffée dans la région abdominale, nous a été fournie par Madame Koprowska de Philadelphie. Il s'agit d'une tumeur d'origine cervicale utérine provoquée par des attouchements au benzopyrène. La tumeur croît d'une manière rapide et possède un caractère histologique stable.

CARCINOME D'EHRLICH (4, 5)

Ce carcinome a été obtenu à partir d'un adénocarcinome mammaire. Les cellules tumorales isolées, injectées par voie intrapéritonéale développent rapidement un cancer ascitique à évolution aigüe; les mêmes cellules, injectées par voie sous-cutanée, donnent naissance à des tumeurs solides très malignes.

L'évolution des tumeurs malignes fut contrôlée par la mesure régulière de la surface tumorale ou par le poids de la tumeur. Les animaux furent pesés régulièrement et une observation clinique journalière nous permit de juger de l'état de santé des animaux traités et des animaux témoins. Chaque expérience fut contrôlée par l'observation d'un nombre au moins égal d'animaux témoins traités de façon identique par sérum physiologique. Nous avons noté la survie en jours et l'apparition éventuelle des métastases. Dans la plupart des cas, un examen anatomo-pathologique fut pratiqué à la mort de l'animal; il comprenait un examen macroscopique de la tumeur et des principaux organes ainsi qu'un examen histologique complet.

Nous avons utilisé, pour le traitement des animaux, un extrait stable de venin de Cobra (*Naja naja atra* et *Naja melanoleuca*). La solution est préparée à partir d'une poudre obtenue par lyophylisation du venin frais, dans une solution isotonique de chlorure de sodium. Ce venin est injecté en général à des doses variant entre ¼ de gamma à 1 gamma. La voie utilisée pour le traitement dépend de la nature de la tumeur:

Les tumeurs solides sont traitées par des injections intratumorales de venin, tous les deux jours, à la dose de 1 gamma en général. Dans certains cas particulièrement résistants, la dose de 5 gamma a été utilisée. Enfin, dans une autre variété de tumeur solide, le carcinome d'Erlich, la voir intrapéritonéales a été essayée.

Les tumeurs ascitiques ont été traitées classiquement par des injections intrapéritonéales à partir du 3e jour qui a suivi la greffe et pendant 6 jours.

Dans le cas du carcinome ascitique d'Ehrlich, nous avons utilisé une méthode consistant à traiter par le venin les cellules cancéreuses *in vitro* avant leur injection aux animaux, en concentrant l'action de faibles doses dans un petit volume. Ce test, s'effectue de la manière suivante: on prélève des cellules cancéreuses d'Ehrlich sur un animal porteur d'ascite. On compte ces cellules et on fait incuber, à 37° durant 3 heures, 1.000.000 de ces cellules en suspension dans le sérum physiologique. On les injecte ensuite par voie intrapéritonéale à des souris.

Le test d'activité du venin est réalisé dans les mêmes conditions, les cellules étant incubées au contact de 1 gamma de venin.

RÉSULTATS EXPÉRIMENTAUX ET DISCUSSION

Tumeurs mammaires spontanées

Pour la facilité de l'expérience, nous avons réparti ces tumeurs en trois classes suivant leur grandeur:

— inférieures à 150 mm²;

— comprises entre 150 et 350 mm²;

— supérieures à 350 mm².

Les résultats obtenus sont repris dans le tableau ci-dessous.

	Surface tumorale		Poids		Survie	
	Témoins (%)	Traités (%)	Témoins (%)	Traités (%)	Témoins (jours)	Traités (jours)
Classe I						
NM 0,5 γ	+ 177	+ 31	− 2	0	24	20
NM 1 γ	+ 177	− 63	− 2	− 6	24	32
NNA 1 γ	+ 177	+ 35	− 2	+ 3	24	20
Classe I sans métastases						
NM 0,5 γ	+ 177	− 13	− 2	+ 10	24	20
NM 1 γ	+ 177	− 63	− 2	− 6	24	32
NNA 1 γ	+ 177	− 12	− 2	+ 3	24	20
Classe I						
Tumeurs 100 mm²	+ 151	− 51				
Tumeurs entre 100 et 150 mm²	+ 223	+ 29				
Résultats réunis						
Sans métastases	+ 177	− 17	− 2	0	24	24
Avec métastases	+ 177	+ 6	− 2	0	24	23
Classe II						
NM 0,5 et 1 γ	+ 62	+ 5	− 6	0	23	24
NNA 1 γ	+ 62	+ 15	− 6	0	23	20
Classe III						
NM 1 γ	+ 15	− 52	− 12	− 11	16	17
NNA 1 γ	+ 15	− 13	− 12	− 13	16	17
Classe II + III	+ 47	− 27	− 7	0	21	21
Classe I + II + III	+ 84	− 20	− 4	− 4	23	22

Par un souci de rigueur, afin de ne pas fausser les statistiques, tous les animaux observés ont été considérés dans ce tableau, même ceux qui ont succombé prématurément et subitement, par choc à la suite d'une injection de venin ou de sérum physiologique.

Ce tableau analyse, en moyennes arithmétiques des valeurs relatives l'évolution des surfaces tumorales, des poids et de la survie.

Il apparaît clairement que les tumeurs des animaux témoins se sont développées d'une manière impressionante, atteignant le triple de la surface primitive. Quant à la plupart des animaux traités, ils montrent un ralentissement net de la croissance, un arrêt, une régression et parfois même une disparition de la tumeur.

La surface tumorale, dans la classe I, des témoins augmente uniformément de près de 177%. Chez les animaux traités, cette surface tumorale subit une régression allant jusque — 63% (1 gamma de venin de *Naja melanoleuca*).

L'augmentation de la surface, des tumeurs dans la classe II des animaux témoins, est moins nette (+ 62%), les tumeurs étant déjà plus grosses à l'origine. Les animaux traités subissent un arrêt total de croissance.

Les tumeurs, dans la classe III, déjà considérables à l'origine ne croissent guère (+ 15%). Malgré cela, le traitement fait apparaître une nette régression allant jusque —52%.

De l'examen des trois classes réunies, on peut conclure que les venins de Cobra ont une action freinatrice et régressive (— 20%, témoins + 84%).

On n'observe pas d'action bien particulière du venin sur l'évolution du poids et la survie par rapport aux témoins. Il faut remarquer à ce sujet que nous avons injecté du venin total et qu'il faut tenir compte de sa toxicité.

L'inhibition et la régression des tumeurs sous l'influence du traitement par le venin total de Cobra est confirmée par l'examen anatomopathologique. Ces examens des coupes des traitées montrent que les zones malignes sont progressivement étouffées par des tissus fibronécrotiques. Les cellules cancéreuses sont dispersées mais encore parfaitement reconnaissables. Une telle image ne s'observe qu'exceptionnellement dans les petites tumeurs témoins. Dans un certain nombre de cas, le tissu cancéreux a complètement disparu. Il est remplacé par du tissu fibronécrotique ou par du tissu inflammatoire. Les modifications des caractères histologiques que nous venons de décrire et qui correspondent à une disparition progressive du tissu cancéreux se retrouvent dans 80% des tumeurs traitées.

Tumeurs greffées solides

Nous avons étudié 3 tumeurs solides dont deux adénocarcinomes et un épithélioma.

Ce dernier s'est révélé insensible à l'action du venin de Cobra. D'ailleurs tous les auteurs anciens avaient fait la même remarque au sujet des tumeurs provoquées par le goudron.

Dans l'adénocarcinome Gaspari, les animaux témoins et traités ont tous été sacrifiés après trois semaines d'observation ce qui correspond au temps d'évolution naturel de la tumeur témoin. Les tumeurs ont été disséquées et pesées. Le tableau témoigne que les poids moyens des tumeurs des animaux traités ne sont que la moitié du poids des tumers témoins. Cette action s'est révélée statistiquement significative après analyse de la variance.

Dans le cas de l'adénocarcinome Mühlbock les tumeurs, ont été traitées par des doses de 1 à gammas de venin total de *Naja naja atra*. A la dose de 1 gamma, aucun effect significatif n'apparaît alors qu'à la dose de 5 gammas une activité se manifeste, statistiquement significative, aussi bien si l'on prend pour critère l'évolution de la surface tumorale que si l'on se base sur le poids de la tumeur.

Le traitement des tumeurs solides d'Ehrlich s'est fait par voie intratumorale et intrapéritonéale à la dose de 1 gamma. Les rapports des moyennes des surfaces tumorales en fin d'expérience sont de 512/316 par voie intratumorale et 512/400 par voie intrapéritonéale. Si l'on prend comme critère le poids des tumeurs, le rapports sont respectivement de 12,1/6,7 et 12,1/7,6. L'analyse de la variance a confirmé l'action freinatrice du venin sur ces tumeurs.

Les tumeurs ascitiques d'Ehrlich ont été provoquées par inoculation intrapéritonéale de 1.000.000 de cellules ascitiques d'Ehrlich à des souris Swiss. Trois jours après l'inoculation, on a injecté 1 à 5 gammas de venin total en répétant cette opération pendant 6 jours. A la dose de 1 gamma, aucune action statistiquement significative n'est apparue et à la dose de 5 gammas, la moitié des animaux a succombé. Dans ce cas également, l'action sur l'ensemble des animaux est peu nette, mais nous avons été cependant frappés par le fait que certains animaux ont survécu jusqu'à 37 jours alors que les témoins n'ont pas dépassé 21 jours.

Dans le test *in vitro*, après l'injection des cellules ascitiques d'Ehrlich, tous les animaux développent de l'ascite à partir du $8^{ème}$ jour et succombent au $20^{ème}$ jour. Contrairement les animaux injectés avec les cellules ascitiques d'Ehrlich incubées au contact avec 1 gamma de venin, ne font jamais d'ascite.

Nous avons vérifié la validité de ce nouveau test en l'appliquant à un chimiothérapique anticancéreux bien connu et bien étudié: l'Actinomycine D, inhibiteur du RNA messager. A la dose de 0,5 gamma, l'Actinomycine D, tout comme 0,5 gamma de *Naja naja atra*, supprime complètement *in vitro* la possibilité d'apparition de l'ascite. Il est intéressant de remarquer que le venin total de *Naja naja atra* est moins toxique que l'Actinomycine D alors qu'il agit à la même dose.

Nous avons appliqué notre test *in vitro* à l'étude de l'action des deux principales substances toxiques du venin de Cobra: la cobratoxine qui nous avait été fournie par le Professeur Yang et la lysolécithinase, isolée par nous.

A des doses croissantes allant jusqu'aux doses sublétales, aucune action anticancéreuse ne fut observée.

La question qui se pose ensuite est de savoir ce que deviennent les cellules d'Ehrlich après l'action du venin total. Nos premières expériences semblent montrer qu'elles ne sont pas simplement détruites comme on pourrait le supposer mais qu'elles restent vivantes et l'examen microscopique révèle qu'elles conservent leurs caractères morphologiques normaux. Leur nombre n'a pas diminué ainsi qu'en témoigne le comptage. Ce travail est actuellement en cours dans notre laboratoire et fera l'objet de la suite de nos recherches dans ce domaine.

Summary

Ten different types of spontaneous or grafted tumors of the Mouse are treated by Cobra venom.

The product is injected by intratumoral and sometimes by intraperitoneal routes, particularly in the case of solid tumors.

Ascitic abdominal tumors are treated by intraperitoneal route. The mammary spontaneous tumors, treated in this way, regress about 20% whereas the tumors of the control group grow 177%.

The anatomopathological examination confirms that there is a resolving action on the cancerous cell due to a fibronecrosis process.

All the solid carcinomas that we studied showed a statistically significative inhibition of their growth.

Isolated Ehrlich's cancerous cells, after treatment with *Naja naja atra* venom failled to produce ascitis when injected in mice.This action of the Cobra venom can be compared to that of Actinomycin D at an equal dose.

The toxic components of the venom (Cobratoxin, lecithinase) showed no activity on the Ehrlich's cell.

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38. 5'-NUCLEOTIDASE ACTIVITY IN SNAKE VENOMS

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5'-nucleotidases, the enzyme capable of hydrolyzing nucleoside 5'-monophosphates to nucleosides and orthophosphate are widely distributed in nature. 5'-nucleotidase activity has been found in animal tissues (1-3), bull semen (4), micro-organisms (5, 6) and in venoms of different species of snakes (7, 8). In human tissues, the highest activity has been found in the posterior pituitary gland (9, 10). Venoms generally exhibit high 5'-nucleotidase activity. This enzyme is found with phosphodiesterase and non-specific phosphatases in venoms which have been largely used in recent years to purify phosphodiesterase and 5'-nucleotidase as these enzymes are valuable tools in the study of structure and nucleotide sequence of nucleic acids (11-14).

After the findings of Gulland and Jackson (7) showed the presence of 5'-nucleotidase in venoms, many attempts had been made to isolate it from venoms in order to study the substrate specificity of the enzyme, its metal requirement, and optimum pH (13-15). We have recently studied 5'-nucleotidase activity in venoms of different species of snakes from widely separated parts of the world and the effects of various metallic ions, inhibitors, and activators on the enzyme activity. The results are presented in this paper.

Methods

In the present study a 20 mg percent venom solution made in glass-distilled water, the pH of which was previously adjusted to 7.4 with 1N $NaHCO_3$ was used. The assay involved the measurement of orthophosphate liberated from adenylic acid (5'-AMP) when a reaction mixture containing 0.1 ml of the venom solution, 5 μmoles of 5'-AMP (Na salt), 1 μmole of Mg^{++} and 20 μmoles of glycine buffer, pH 8.5, in a total volume of 0.5 ml, was incubated at 37°C for 20 min. The reaction was terminated by adding an equal volume of 10% cold trichloroacetic acid (TCA), and allowing it to stand at 0°C for 15 min and then centrifuging it at 1200 × g at 0°C for 15 min. The clear liquid (TCA extract) was used for the orthophosphate determination. The methods of Fiske and Subba Row (16), Cleland and Slator (17) were used to measure Pi and protein.

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Results

Fig. 1 shows the 5'-nucleotidase activity of six venoms obtained from three different species of snakes. The rate of hydrolysis of 5'-AMP was linear to the enzyme concentration in each case, until 65 to 70% of the substrate was hydrolyzed. Of all the venoms tested for 5'-nucleotidase activity the venom of *Crotalus adamanteus* exhibited highest activity and the venom of *Bothrops jararaca* the least. The venom of *Vipera russelli* showed slightly less activity than the venom of *C. adamanteus*. The Km values calculated for the venoms of *V. russelli* and water moccasin by Lineweaver and Burk's equation (18) from the plots 1/V against 1/S (Fig. 2) were found to be 2.2×10^{-3} and 1.66×10^{-3} M.

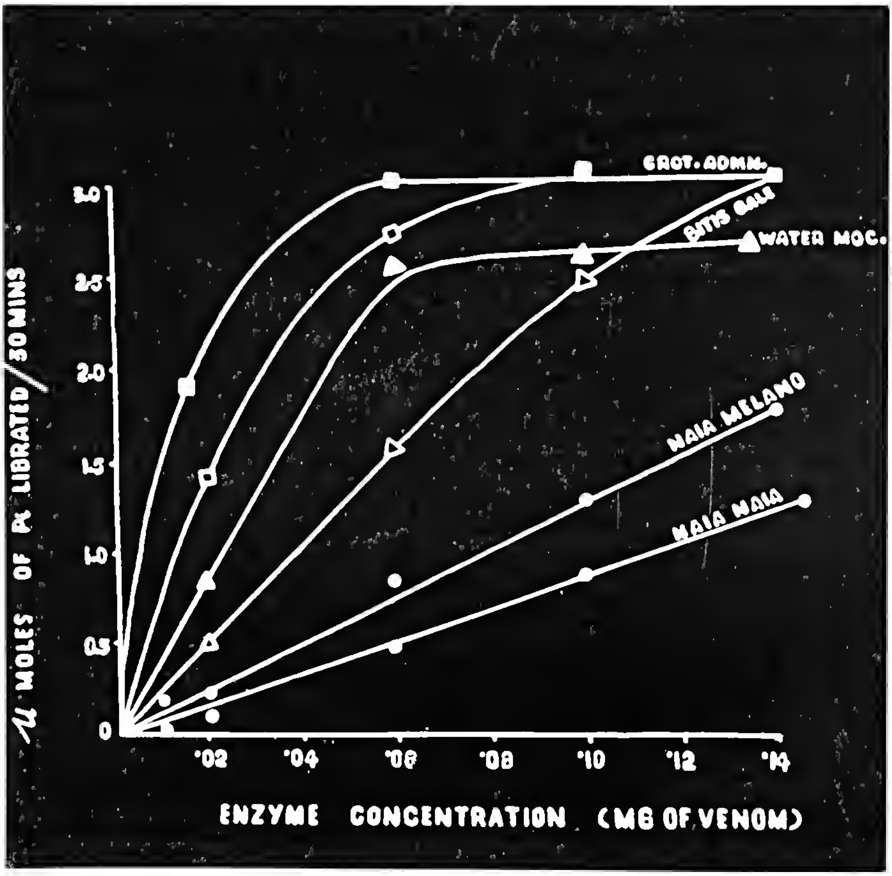

Fig. 1

Effects of metallic ions, ortho- and pyrophosphates and reducing agents on venom 5'-nucleotidase activity.

Maximum activity was found in the presence of Mg^{++}; and in its absence 60-70% activity was noted. In the presence of EDTA, the activity dropped to 10-15%. However, when Mg^{++} was added a twofold increase in activity was found. With dialyzed venom which showed only 20-22% of the activity of

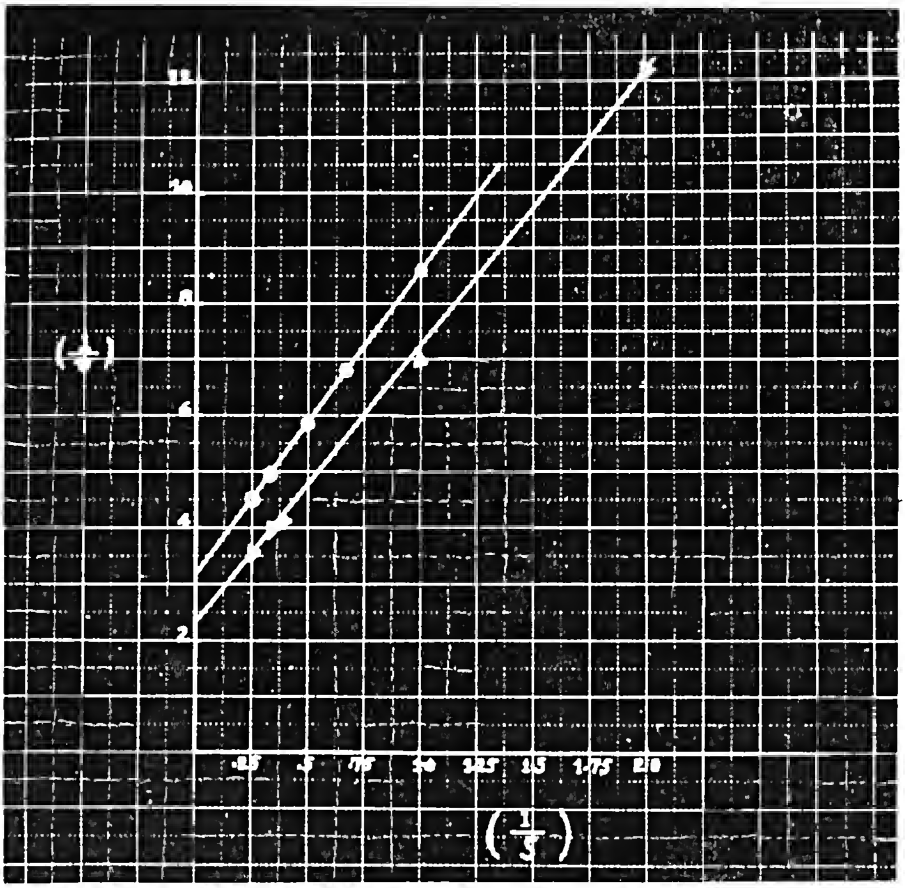

Fig. 2

the undialyzed venom, a three- to fourfold increase in activity was observed when the activity was measured in the presence of both EDTA and Mg^{++}. Zn^{++}, Mn^{++}, and Ni^{++} inhibited the enzyme activity strongly. Co^{++} at low concentrations stimulated the enzyme activity and at high concentrations inhibited its action (Table I).

Orthophosphate and pyrophosphate both inhibited 5'-nucleotidase activity. Cystein and glutathione enhanced the enzyme activity, but methionine and cystine inhibited its activity. NaF acted as an inhibitor but was less active than the orthophosphate and pyrophosphate (Table I).

Effects of pH on venom 5'-nucleotidase

From the pH-activity curve (now shown) it was concluded that venom 5'-nucleotidases have no well-defined pH optimum because the venom of *V. russelli* exhibited maximum activity over the pH range 7.6 to 8.4, and *C. adamanteus* venom was equally active over the pH range 8.0 to 10.0. At pH below 7.4

TABLE I — EFFECTS OF METALLIC IONS, ACTIVATORS AND INHIBITORS ON VENOM 5'-NUCLEOTIDASE ACTIVITY

Reagents		μmoles added	Relative Activity	Activation (%)	Inhibition (%)
Complete system		—	100	—	—
"	$- Mg^{++}$	—	70	—	—
"	$+ Mn^{++}$	0.05		—	75
		0.10		—	94
"	$+ Ni^{++}$	1.00		—	70
		2.00		—	82
"	$+ Co^{++}$	0.10		20	
		1.00		—	12
"	$+ Zn^{++}$	5.00			22
		0.05		—	80
		0.10		—	95
"	+ cysteine	1.00		23	—
		5.00		48	—
"	+ glutathione	1.00		18	—
		5.00		46	—
		1.00		—	12
"	+ Pi (Na-phosphate)	2.00		—	37
		5.00		—	88
"	+ PP (Na-pyrophosphate)	1.00		—	52
		2.00		—	71
		5.00		—	96
"	+ NaF	1.00		—	5
		2.00		—	12
		5.00		—	66

The complete system contained 20 μmoles of glycine buffer, pH 8.5, 1 μmole of Mg^{++}, 5 μmoles of AMP (Na-salt) and 0.1 ml of a 0.02% venom solution. The reaction mixture was incubated for 20 mins. at 37°C. The amount of metallic ions, activators and inhibitors added are shown in column 2. The degree of activation and inhibition of 5'-nucleotidase is expressed as the percentage of the activity of the complete system, which has been arbitrarily taken as 100%.

and above 8.4, the 5'nucleotidase activity of *V. russelli* venom declined rapidly. A sharp drop in 5'-nucleotidase activity of *C. adamanteus* venom was also noted at pH below 8.0 and above 10.0.

Substrate specificity of venom 5'-nucleotidase

The ability of venom 5'-nucleotidase to hydrolyze other nucleotides is shown in Table II. The results indicated that venom 5'-nucleotidase can hydrolyze almost all nucleoside 5'-monophosphates and among them, 5'-AMP was hydrolyzed maximally (100%) and 5'-CMP by 76%. Next to it in order of effectiveness as

TABLE II — SUBSTRATE SPECIFICITY OF VENOM 5'-NUCLEOTIDASE

Substrates	Relative Activity
5'-AMP	100
5'-UMP	36
5'-CMP	76
5'-GMP	28
5'-IMP	22
5'-dAMP	35
5'-dTMP	58
5'-dCMP	54
5'-dGMP	26
2'-AMP	5
3'-AMP	8
cyclic-2', 3'-AMP	0
ribose-5'-phosphate	0
α — glycerophosphate	6
β — glycerophosphate	8

The reaction mixture contained 20 μmoles of glycine buffer, 8.5, 1 μmole of Mg^{++}, 5 μmoles of substrate (Na salt), and 0.1 ml of a 0.02% venom solution in a total volume of 0.5 ml, incubated for 20 mins. at 37°C.

substrates were 5'-UPM, 5'-GMP and 5'-IMP which were hydrolyzed by 36, 28 and 22%, respectively. Venom 5'-nuclcotidase can also hydrolyze deoxynucleoside 5'-monophosphates viz 5'-dTMP could be hydrolyzed by 58%, 5'-dCMP, 5'-dAMP and 5'-dGMP by 54, 35 and 26%, respectively. Neither ribose-5-phosphate nor cyclic-2',3'-AMP could be hydrolyzed. Very limited hydrolysis was noted with α- and β-glycerophosphates.

Discussion

The twofold increase in the activity of cobra venom 5'-nucleotidase observed by Kaye (19) after dialysis, was not noted by us with the venom of *V. russelli*, but a three- to fourfold increase in the activity of the dialyzed venom was noted when measured in the presence of EDTA and Mg^{++}. Probably the venom used by us did not contain any free Zn ions or other poisonous metals that can be removed by dialysis, but contained traces of metals in bound form that can be removed by EDTA. This perhaps explains the fourfold increase in activity noted by Kaye (19) and us when the activity was measured in the presence of EDTA and Mg^{++} and not just in the presence of Mg^{++} alone. A 90% inhibition

of enzyme activity was observed with orthophosphate and pyrophosphate whereas NaF inhibited only 65% of the activity. Activation by glutathione (reduced form) and cysteine amounted to 46 and 48% in each case (Table II).

Because venoms rich in 5'-nucleotidase activity did not hydrolyze α- and β-glycerophosphates at pH 5.0 and 9.0 and their activity was inhibited by Ni^{++}, which did not affect the hydrolysis of glycerophosphates by nonspecific phosphomonesterases, the hydrolysis of nucleoside 5'-monophosphates by 5'-nucleotidase cannot be related to the action of any nonspecific phosphomonesterase present in the venoms. It can not either be linked to venom adenosine triphosphatase since the hydrolysis of ATP by venoms was not altered by Co^{++}, which enhanced the hydrolysis of 5'-AMP by venoms at low concentrations and inhibited at higher concentrations. Venom 5'-nucleotidase was not identical to 5'-nucleotidases obtained from other sources since the hydrolysis of 5'-AMP by them was not affected in the same way by the same metallic ions and also because the pH at which maximum hydrolysis of the substrate attained, was also different. Although venom 5'-nucleotidase hydrolyzed all deoxynucleoside 5'-monophosphates such as 5'-dAMP, 5'-TMP, 5'-dCMP, and 5'-dGMP the affinity of the enzyme for 5'-AMP was much greater than for 5'-dAMP. Other deoxy compounds were also hydrolyzed but to lesser degrees than the corresponding ribose compounds. The hydrolysis of deoxy nucleotides by bacterial 5'-nucleotidase was first reported by Carter (20). It did not however hydrolyze 2'-AMP, 3'-AMP or cyclic-2',3'-AMP, nor catalyze the hydrolysis of ribose-5-phosphate. 5'-nucleotidases obtained from different sources appear to be different in many ways, particularly in regard to their behaviour towards metallic ions and inhibitors, and pH, their substrate specificity seems to be the same, and therefore should be considered as isoenzymes.

The existence of an enzyme capable of hydrolyzing a specific substrate (adenylic acid), and as widely distributed in nature as its own substrate possibly implies the involvement of this enzyme in regulating the concentrations of orthophosphate for glycogenolysis and adenylic acid (other monophosphates) for nucleic acid synthesis in the cell. It is difficult to understand the reason for the high 5'-nucleotidase activity in snake venoms since the amount of enzyme present is not sufficient to inflict any toxic effect. It may be possible that the enzyme 5'-nucleotidase, like other enzymes present in the venoms, also contribute to a limited extent towards the total toxicity of the venoms (21).

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V

FARMACOLOGIA

PHARMACOLOGY

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39. CONSTITUENTS OF THE VENOM OF THE AUSTRALIAN BULL ANT, *MYRMECIA PYRIFORMIS*

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Preliminary studies (1, 2), on the venom of an Australian bull ant, *Myrmecia pyriformis* (previously identified as *M. forficata*) revealed the presence of high concentrations of histamine (1·3% by weight of the dried venom) and another substance possessing smooth muscle stimulating properties. This latter component, separated from the histamine by means of ascending paper chromatography causes a slow, persistent contraction of the mepyramine-treated guinea pig ileum and of the virgin rat uterus, and produces a prolonged hypotensive response in the anaesthetized cat, distinct from the rapidly reversible depressor response to histamine. More recently Cavill *et al.* (3) have reported in the venom of a related species of ant, *M. gulosa*, the presence of hyaluronidase activity and a direct haemolytic component, in addition to histamine and a smooth muscle stimulant resembling that described for *M. pyriformis*. They achieved partial separation of the components by means of low voltage paper electrophoresis although this technique did not permit separation of the hyaluronidase from the smooth muscle stimulant.

Subsequently, we have shown that the crude venom of *M. pyriformis* possesses hyaluronidase activity (determined turbidimetrically by the method of Tolkesdorf *et al.*) (4) and the ability to lyse washed red blood cells using a modification of the method described by Neumann, Habermann & Hansen (5). I mg of the crude venom was found to be equivalent to 100 IU of hyaluronidase (Rondase) and is therefore 10 times as potent as commercial testicular hyaluronidase on a weight basis. Activity could be demonstrated in 10 μg crude venom.

On account of the prolonged nature of the local responses in man to sting by *M. pyriformis*, an investigation was made into the possibility that the venom contains components which might act indirectly through the endogenous release of pharmacologically active substances such as histamine and bradykinin. The crude venom was tested for its ability to release histamine from isolated rat mast cells (6) according to the technique described by Rothschild (7), and its potency in this respect was compared with 48/80. Saline extracts of venom or solutions of 48/80 were incubated with cells from the peritoneal fluid of rats and after incubation the residual histamine content of the cells was assayed on the guinea pig ileum. Activity was expressed in terms of % release. As little as 10 μg of venom was found capable of releasing appreciable amounts of histamine and this dose was roughly equivalent to 2.5 μg 48/80. In some experiments activity was demonstrated by allowing live ants to release venom directly into the incubating fluid containing the mast cells. Although the method was not sufficiently sensitive to show any response from 1 sting, the venom of 10 insertions of a sting resulted in considerable histamine release.

The method used for the detection of bradykinin-releasing activity was based on that described by Margolis *et al.* (8). Saline extracts of the venom were incubated with bradykinin-free rabbit plasma in the presence of EDTA and the bradykinin-released was assayed on the rat duodenum. In some experiments, suspensions of human bradykininogen replaced the samples of rabbit plasma and in all experiments comparisons were made with trypsin. Concentrations of venom 30 times those capable of producing histamine release showed no evidence of bradykinin-releasing activity.

Further characterization of these pharmacologically active components has been attempted by the application of fractionation procedures, boiling and digestion with proteolytic enzymes. By these means it has been possible to conclude that the various activities can be attributed to separate substances, with the exception of the haemolytic component which it has not been possible to separate from the smooth muscle stimulant.

Ascending paper chromatography was carried out in water saturated butanol acetic acid (5:4:1), these conditions having been used to separate the histamine from the smooth muscle stimulant in the preliminary studies. Chromatograms were run for 24 hrs after which 1/2″ strips were eluted in saline and eluates submitted to tests for pharmacological and enzymic activity. Hyaluronidase activity disappeared altogether under these conditions, histamine was clearly separated from all other activities, but there was considerable overlap of the histamine releasing, smooth muscle stimulant and haemolytic components. However, a high percentage of the histamine releasing activity had a Rf value close to zero and it appears that a separate component is responsible for at least part of the histamine releasing activity of the venom. The partial separation of this factor from the other active components by means of low voltage paper electrophoresis at pH 6.24 again suggests that there may be two fractions contributing to histamine release, one of which is a distinct component and the other indistinguishable from the smooth muscle stimulant.

Electrophoresis was carried out at 4°C and a pH of 6.24 as described by Cavill *et al.* (3). A current of 0.3 mamps/cm was applied for 16 hours after which the paper was divided into 1″ strips, eluted in saline and tested for the various activities. Hyaluronidase and the smooth muscle stimulant could not be separated and in our experiments the haemolytic activity moved towards the cathode parallel to the smooth muscle stimulant. This is in contrast to the haemolytic component of *M. gulosa* which is reported to move towards the anode.

Dialysis through Visking cellophane membranes was carried out for 24 hours at 4°C with the ratio of inner to outer volume being 1:10. After dialysis, the volume of the dialysate was reduced 10 fold by freeze drying and redissolving in the appropriate amount of distilled water.

With the exception of histamine which was always 100% dialysable, the components were only slowly dialysable and their rates of dialysis were somewhat variable. However histamine releasing activity and the smooth muscle stimulant were always in detectable concentrations whereas hyaluronidase and haemolytic activities were sometimes less than 10% dialysed, and could not be detected. The behaviour of each component on chromatography, electrophoresis and dialysis is summarized in the Table I. Table II shows the percentage activity of each component remaining after digestion with trypsin, chymotrypsin and boiling for 10 minutes at pH 6.0.

TABLE I *

COMPONENT	PAPER CHROMATO-GRAPHY Rf value	ELECTRO-PHORESIS Rate of move-ment to Catho-de: ins/16 hours	DIALYSIS % Dialysed in 24 hours
Histamine	0.22 (0.18 — 0.26)	5.25 (4.5 — 6.5)	90% (3)
Smooth muscle stimulant	0.14 (0.13 — 0.15)	1.3 (0.5 — 2.5)	13 — 30% (3)
Histamine release	0 — 0.15	2.25 (1.5 — 3.5)	36% (1)
Hyaluronidase	—	1.25 (0.5 — 2.5)	Undetectable < 8% (2)
Haemolytic	0.14 (0.11 — 0.17)	2.0 (1.5 — 2.5)	Undetectable < 12% (1)

* Figures in brackets refer to number of separate determinations.

TABLE II *

COMPONENT	BOILING pH 6.0 % Activity remaining	TRYPSIN DIGESTION % Activity remaining	CHYMOTRYPSIN DIGESTION % Activity remaining
Histamine	100% (3)	100% (3)	100% (1)
Smooth muscle stimulant	37% (3)	10% (4)	50% (2)
Histamine release	100% (2)	10 — 20% (4)	30 — 40% (2)
Hyaluronidase	3% (3)	20 — 67% (3)	—
Haemolytic	31% (2)	10% (1)	—

* Figures in brackets refer to number of separate determinations.

The various procedures outlined above shed light on some characteristics of the various components of the venom of *Myrmecia sp.* although detailed characterization of the constituents requires more satisfactory methods of fractionization. In many respects the venom resembles that of the bee and has constituents belonging to each of the three classes described by Habermann (9) for **HYMENOPTERA** venoms — namely biogenic amines, peptides and non-enzymic proteins, and enzymes, — typified by histamine, the smooth muscle stimulant and hyaluronidase respectively.

The smooth muscle stimulant fraction is slowly dialysable through Visking cellophane membranes and is only partially destroyed by boiling. However, almost all the activity is lost by incubation with trypsin. It contracts the smooth muscle of the guinea pig ileum, the virgin rat uterus and the blood vessels of the

isolated rabbit ear. Tachyphylaxis occurs, but least markedly in the perfused rabbit ear and consequently this preparation, treated with mepyramine, has been used most frequently, doses being administered at intervals of 20 minutes. An example of this is shown in Fig. 1. Activity has been demonstrated with 10 μg of crude venom. There has been no clear-cut distinction between this factor and the haemolytic component and it is possible that the smooth muscle stimulant possesses haemolytic properties. It may also account for some of the histamine releasing activity.

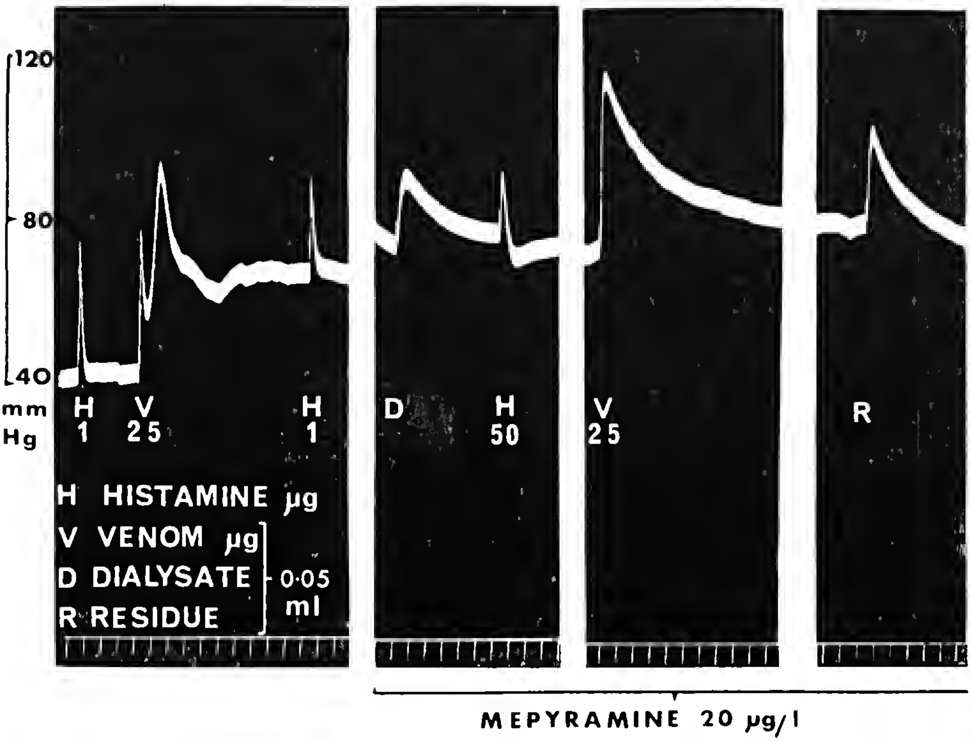

Fig. 1 — Isolated Rabbit Ear perfused with Kreb's solution at 37°C. Perfusion rate 7 mls/min. Constriction recorded by rise in perfusion pressure. Dialysate and residue derived from a 25μg equivalent of venom dialysed for 24 hours. Time scale — 1 minute.

Phospholipase A is widely distributed among insect and animal venoms and it is conceivable that the venom of *Myrmecia sp.* also contains this enzyme, and if this is so, it could contribute to the histamine releasing activity. In this conection attention is drawn to two properties of Phospholipase A — namely its heat stability and its ability to dialyse slowly, both of which apply to the histamine releasing activity of the ant venom. Phospholipase A and lysolecithin themselves possess various pharmacological activities including smooth muscle stimulation, a delayed and prolonged hypotensive effect and increased capillary permeability — all of which are properties possessed by the venom. Larger scale separation and attempts at preparation of relatively pure fractions are in progress, and it is hoped to embark on a more detailed analysis of the components, particularly the smooth muscle stimulant and the histamine releasing factor as soon as purer fractions become available.

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40. RECENT ADVANCES ON THE MECHANISM OF ACTION OF BRAZILIAN SCORPION VENOM — *T. SERRULATUS*

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The mechanism of the pronounced systemic hypertension produced by scorpion venom is not yet completely understood. Most of the theories relate the hypertensive response to a sympathetic stimulation, which, according to the different authors could be: *peripheral* (1, 2) (post ganglionic endings of the sympathetic system), *central* (3) (spinal cord and sympathetic preganglionic neurons) or both (4, 5). However, no attempt was made to suggest whether the sympathetic stimulation produced by the venom is a direct one or mediated by released catecholamines. On the other hand, it is known that the venom induces a contraction of the guinea-pig ileum through the release of acetylcholine (6, 7).

Since the venom seems to interfere with both components of the autonomic nervous system, we decided to investigate its actions on the isolated guinea-pig heart which gives opposite responses whether adrenergic or cholinergic agents are tested.

Isolated hearts from eighteen guinea-pigs prepared according the Langendorff method, were used.

The typical effect of the venom * is a bradycardia followed by a conspicuous increase in the force and frequency of the heart beatings; these effects are achieved in doses ranging from 5-10 mcg which to correspond to a final concentration of the order of 2×10^{-6} to 1×10^{-6} g/ml. In a few hearts a higher dose (20-40 mcg) was necessary to produce the bradycardia which usually precedes the stimulating effect (Fig. 1). We have shown that the cardiostimulating effect is indirect and due to the release of catecholamines since it was blocked by D.C.I. (9) and bretylium (2×10^{-5} g/ml/min.) and was absent in the reserpine treated animals ** (Fig. 2).

In our experiments no potentiation of the cardiac effects of epinephrine by the venom was seen indicating no interference by the venom in the mechanisms of inactivation of catecholamines.

The bradycardic response to the venom is better seen when the cardiostimulating effect is blocked by D.C.I. bretylium or previous reserpinization. The bradycardia is cholinergic in nature since it is blocked by atropine (2×10^{-5}

* The whole venom, kindly supplied by Dr. W. Bücherl — Instituto Butantan, São Paulo, was obtained by electrical stimulation(8).

** Three guinea-pigs received 2 mg/kg of reserpine divided in two doses of 1 mg given intraperitoneally 48 and 24 hs. before the experiments.

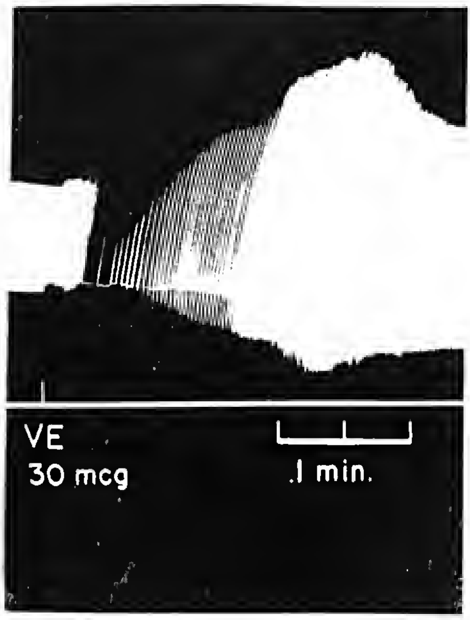

Fig. 1 — Normal guinea-pig heart — Effect of 30 mcg of scorpion venom (*VE* — final concentration 6×10^{-6} g/ml) on the isotonic force of contraction and frequency of heart beating. Notice the bradycardia preceding the cardiac stimulation.

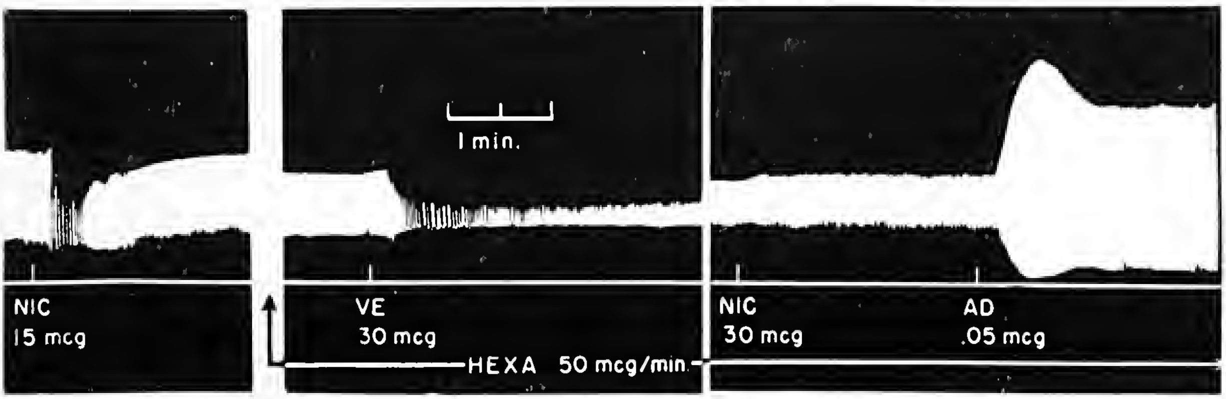

Fig. 2 — Reserpinized guinea-pig heart — No cardiac stimulation is observed with 15 mcg of nicotine (*NIC* — final concentration 3×10^{-6}) or 30 mcg scorpion venom (*VE* — final concentration 6×10^{-6}). Notice that hexamethonium (1×10^{-5} g/ml/min) blocks the bradycardic effect of nicotine but not that of the venom. At *AD*, epinephrine (final concentration 1×10^{-8} g/ml).

g/ml) and potentiated by prostygmine (4×10^{-6} g/ml). Moreover, the bradycardic action of the venom seems to be indirect since it is significantly reduced by hemicholinium (4×10^{-5} g/ml/min.) in agreement with this view is the observation that the contraction of the guinea-pig ileum elicited by the venom is markedly depressed by morphine (6) and cocaine (10). The site of action of the venom is post ganglionic since it was not affected by hexamethonium (1×10^{-5} g/ml/min.) in doses which abolished the response to nicotine (Fig. 2).

The site of action of the cardiostimulating effect of the venom is not at the sympathetic ganglion because it was not affected by either hexamethonium or atropine. On the other hand the effect of the venom is bigger and more resistant to tachyphylaxis than that elicited by tyramine; also the effect of the venom but not of tyramine is blocked by hemicholinium (4×10^{-6} g/ml/min.).

It should be noted that the only drug able to block simultaneously the cholinergic and the adrenergic effects of the venom is hemicholinium, which is a compound known to interfere with the synthesis of acetylcholine (11). Such result strongly suggests the participation of acetylcholine in both effects of the venom, i.e., bradycardia followed by cardiac stimulation. This hypothesis is strengthened by our observation that prostygmine 1×10^{-7} g/ml is able to potentiate the cardiac stimulation in the atropinized heart (Fig. 3). When we consider all these facts they agree with and give further support to the theory proposed by Burn and Rand (12) of the existence of a "cholinergic link" at the sympathetic nerve endings. According to the theory of Burn and Rand, the sympathetic post ganglionic stimulation produces a small discharge of acetylcholine which in turn releases norepinephrine.

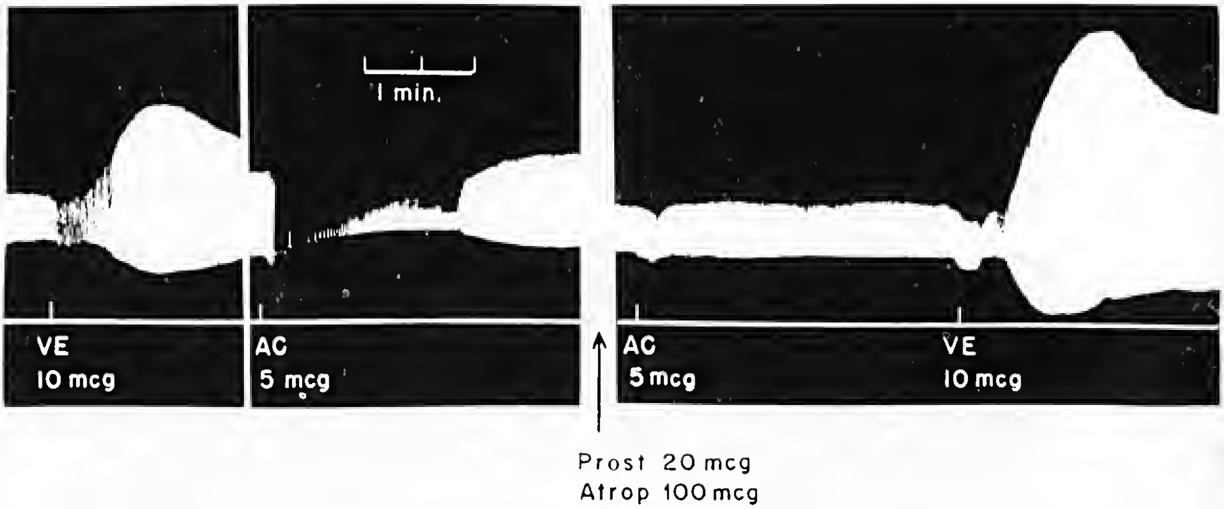

Fig. 3 — Normal guinea-pig heart — Cardiac effects of 10 mcg of scorpion venom (*VE* — final concentration 2×10^{-6} g/ml) and acetylcholine (*AC* — final concentration 1×10^{-6} g/ml) before and after the simultaneous administration of prostygmine (4×10^{-6} g/ml) and atropine (2×10^{-5} g/ml). Notice the increase of the cardiostimulant effect of the venom after treatment of the atropinized heart by prostygmine.

Therefore, the cardiac stimulating effect of the scorpion venom resembles that of sympathetic nerve ending stimulation in the same way as it has been described and assumed for guanethidine (13).

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41. EFFECTS OF SCORPION VENOM AT NEUROMUSCULAR JUNCTION

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The squeletal muscle activity provoked by scorpion venom from mexican *Centruroides* is due to effects on spinal motoneurons and neuromuscular junctions (1).

The latter is a local action because the venom did not activate either denervated muscle or muscular nerves when directly applied.

Mechanograms of muscular responses to single shock stimulations in cats and other mammals under scorpion venom intoxication render bigger and longer contractions than those obtained in normal animals (Fig. 1).

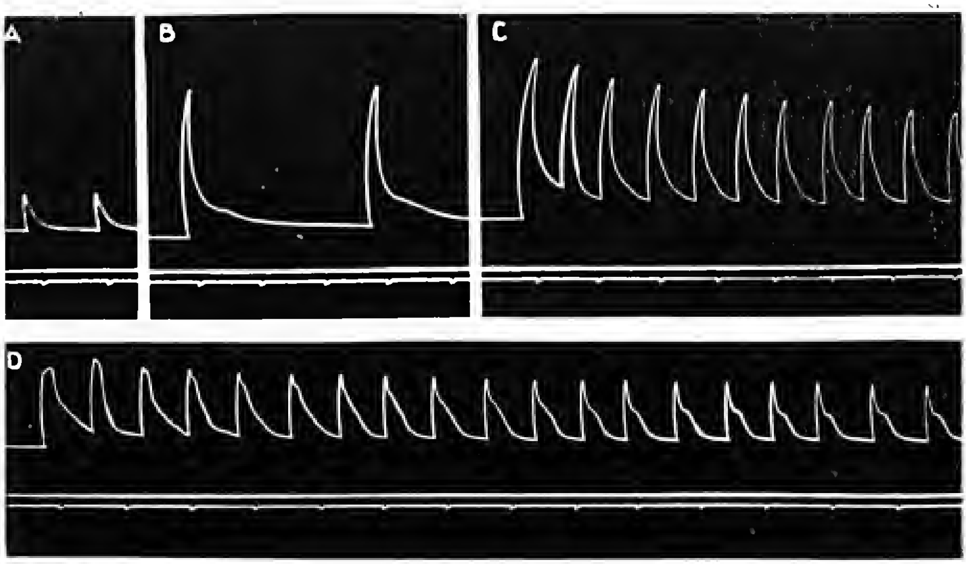

Fig.1 — Increase in amplitude and duration of muscular responses to maximal stimuli under the action of scorpion venom (B). A, control before the venom; C and D, decrease produced by the repetition of responses. (del Pozo and Auguiano (1).

Simultaneous electrical recordings from the muscle and its centrally cut nerves show series of potentials in fast sequence after such single shocks are applied to the nerves. If the nerve is severed from the muscle the repetitive activity suddenly disappears and only the normal action potentials persist (2) (Figs. 2 and 3).

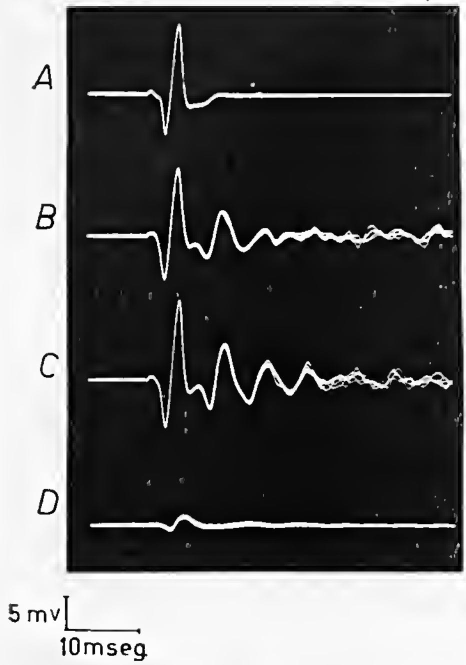

Fig. 2 — Repetitive activity as shown by electromyogram. Several responses superimposed. Stimuli applied to the nerve. A, before the venom; B to D, after the injection of succesive doses of scorpion venom.

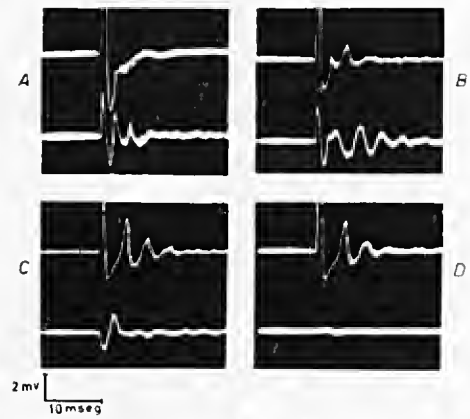

Fig. 3 — Simultaneous recording from responses of sciatic nerve (above) and gastrocnemius muscle (below) to stimuli applied to the nerve. A, before; B, after the injection of a large dose of scorpion venom; C, 5 minutes later; D, after curare.

The present study was carried out with the purpose of analyzing the origin and propagation of the described repetitive electrical potentials.

In cats anesthetized with phenobarbital, electrical records were taken from gastrocnemius muscles and from single fibers of the ventral spinal roots at L^7. The roots were centrally cut. Electrical stimuli were applied to the sciatic nerve. The scorpion venom from *Centruroides suffusus suffusus* Pocock was injected intravenously or directly into the recorded muscle.

The repetitive potentials appeared first in the muscle and after several seconds or in some cases minutes, in the anterior roots. When the sciatic nerve was peripherally cut the repetitive discharges, disappeared. In several experiments recordings electrodes were applied to the posterior spinal roots but no potentials were found (Fig. 4).

According to these results the antidromic propagation of the repetitive impulses by the efferent fibers may be affirmed. The single fiber records leaves no doubt about the repetitive electrical activity in the functional unit.

In other series of experiments, the effect of the venom on the end-plate potential was studied. Silver macroelectrodes were applied to the region of the end-plates of gracilis muscle of cats previously curarized.

The recorded potential was local and propagation with a great decrement was limited to a very short area of the muscle. Under scorpion venom the electrogram showed an initial fast component probably corresponding to immediate nerve terminals which was followed by a negative wave prolonged for about

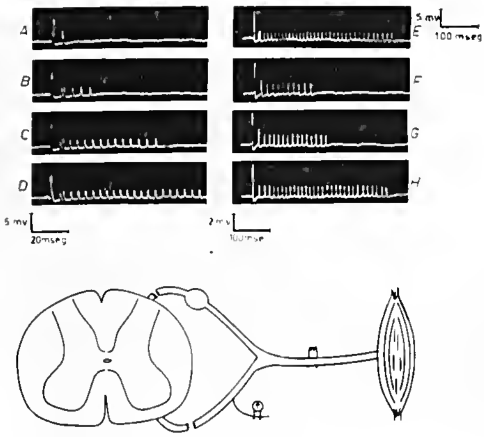

Fig. 4 — Spontaneous variation of the discharges to peripheral single shocks applied to the sciatic nerve after scorpion venom. Recordings from a thin ventral root filament at different intervals.

15 to 20 msec. With succesive doses of the venom this long potential gradually changed in polarity and increased in duration to about two fold the initial time (Fig. 5).

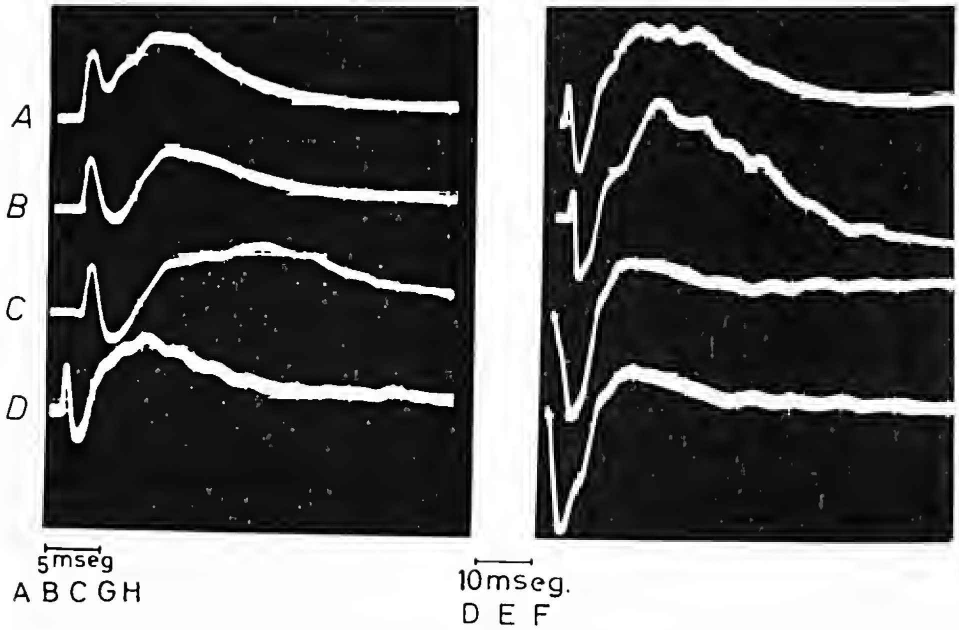

Fig. 5 — Macroelectrodes recordings from end-plate region. Gracilis of cat under the action of scorpion venom. Records from succesive stimuli at different times after the injection of the venom.

Large doses of venom blocked the neuromuscular transmission. This block ocurred many times when the electrical discharges in the nerve were very marked or even increased at the time of the block.

In some recent experiments intracellular recordings of the end-plate potential were done in frogs in order to determine the place of action of scorpion venom. Neuromuscular transmission was previously blocked by curare. The venom did not produce significant changes in the end-plate potential either in amplitude or duration. When the dose of venom was increased the end-plate potential suddenly disappeared. It was tested that these negative records were taken with the intracellular electrode in its place as it was shown by the resting potential appearing when the electrode was taken out (Fig. 6).

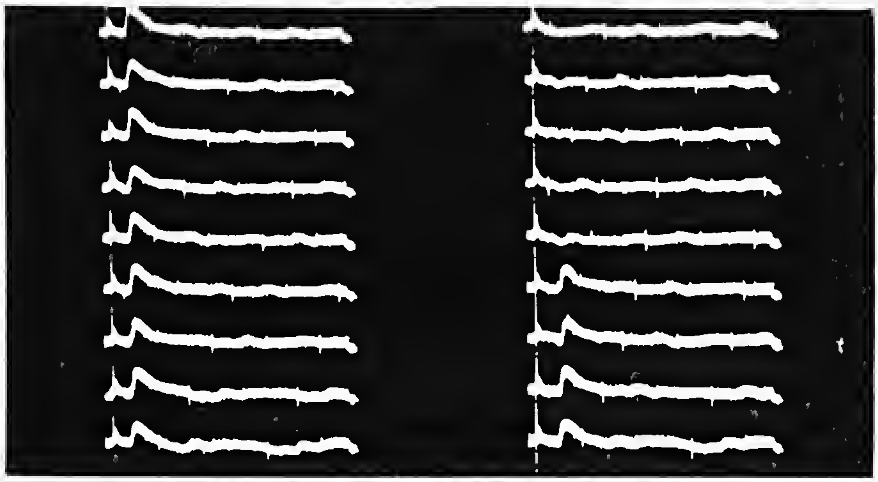

Fig. 6 — Intracellular reeordings from frog sartorius muscle under the action of scorpion venom. Successive responses to shocks applied to the nerve at 2 seconds intervals. The records are to be read from the lower to the upper part, left column first.

Edwards working in this laboratory by measuring the quantal release of the transmitter from the pre-synaptic nerve terminals in frogs under the action of scorpion venom found evidence that this action takes place at the nerve endings.

The last two series of experiments described suggest that the action of scorpion venom at the neuromuscular junction corresponds to activity at the nerve terminals. This activity could explain the repetitive discharges in muscle and nerve previously described.

The extracellular recording of end plate potentials are always difficult to analize because of the complex spatial arrangement of the elements that contribute to the potential registered.

However, is should be kept in mind that the intracellular recordings were done in frogs and the rest of the experiments correspond to cats and other mammals. There is always possible differences due to the species chosen.

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42. ON THE ACTION OF BUFOGENINS AND ALLIED COMPOUNDS ON THE INTESTINE AND OTHER SMOOTH MUSCLE ORGANS

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Pharmacological studies on the active principles isolated from Ch'an Su, the dried venom of the Chinese toad, revealed some interesting activities. For example, bufalin, a coprostane-type steroid with a six membered unsaturated lactone, has a very strong local anesthetic action and, as Dr. Okada reported in this Symposium, its mode of action differs markedly from that of cocaine and resibufogenin, a steroid with an expoxide in 14-15 position, which has a strong stimulating action on respiration and blood pressure. This paper reports the result of an investigation on the pharmacological actions of bufogenins which were mainly on guinea-pig smooth muscles (intestine, blood vessels and trachea), with the expectation of obtaining some useful data which could throw some more light on their action mechanisms.

Their effects on intestinal tissues were principally studied by using the ileum preparation according to the Magnus perfusion method. The resibufogenin minimum effective doses for intestinal contractions were 5×10^{-8} to 10^{-7}, and the dose-response curve was a sigmoid up to the concentration of 4×10^{-6} (Fig. 1), in higher concentrations, the responses were reduced. All bufogenins studied act similarly upon this smooth muscle preparation.

This contracting action on the ileum preparation is depressed to some degree by atropine (10^{-8}) and morphine (5×10^{-7}), and enhanced by eserine (10^{-9}) (Fig. 2), suggesting the participation of Ach release in the concentration mechanism. As it is not influenced by C6 the Ach releasing effect seems not be ganglionic, but post ganglionic (Fig. 2).

The guinea-pig ileum inhibiting effect induced by atropine is not reproduced in the taenia-coli preparation. In the latter preparation, the contracting actions of the so called Ach-releasers such as nicotine and picric acid were not observed (Fig. 3). This seems to indicate, therefore, that its irresponsiveness seems to result on account of its lack of nerve tissues, which release Ach.

The kymogram of Fig. 4 shows the intestinal contractions produced by bufalin and the blocking effects of atropine. The dose-response curves obtained with the same preparation (Fig. 4) show that bufalin has a much more stronger activity than resibufogenin, and that g-strophanthin is less active than resibufogenin. Though digitoxigenin is more active than resibufogenin the latter is more active than digitoxin. Generally the actions of genins are stronger and faster in appearance than those of the glycosides.

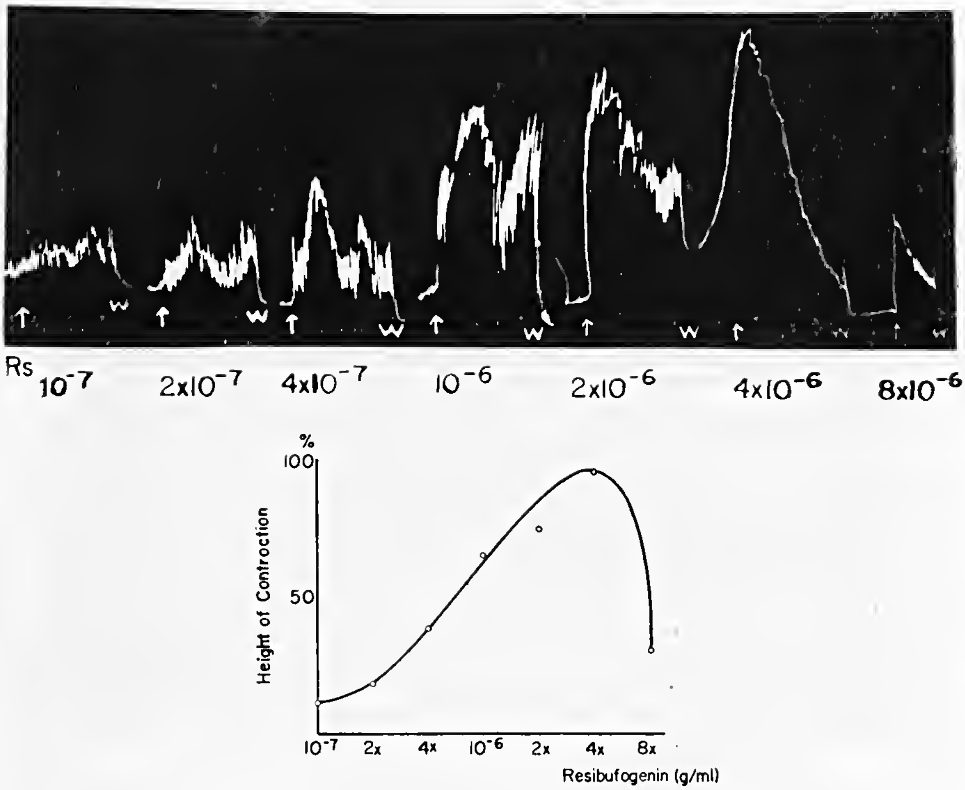


Fig. 1 — Action of Resibufogenin on the small Intestine of Guinea-pig.

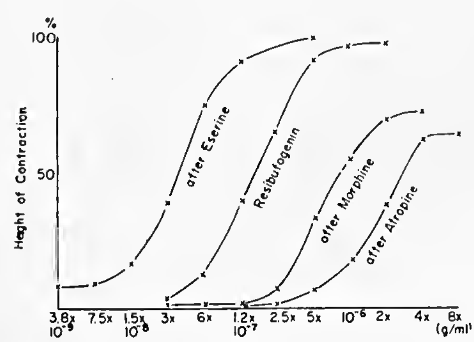


Fig. 2 — Dose-Response Curve of Resibufogenin.

When a comparatively high concentration of resibufogenin is applied to the guinea-pig ileum, relaxation follows contraction considerably faster than with the taenia-coli preparation. Supposing that a probable Ca^{++} release would participate in this mechanism, we studied the effects of nethalide (β-blocker), reserpine and dibenzyline on the mode of relaxation (Fig. 5). There was no remarkable difference of the effects after the pretreatment by these substances.

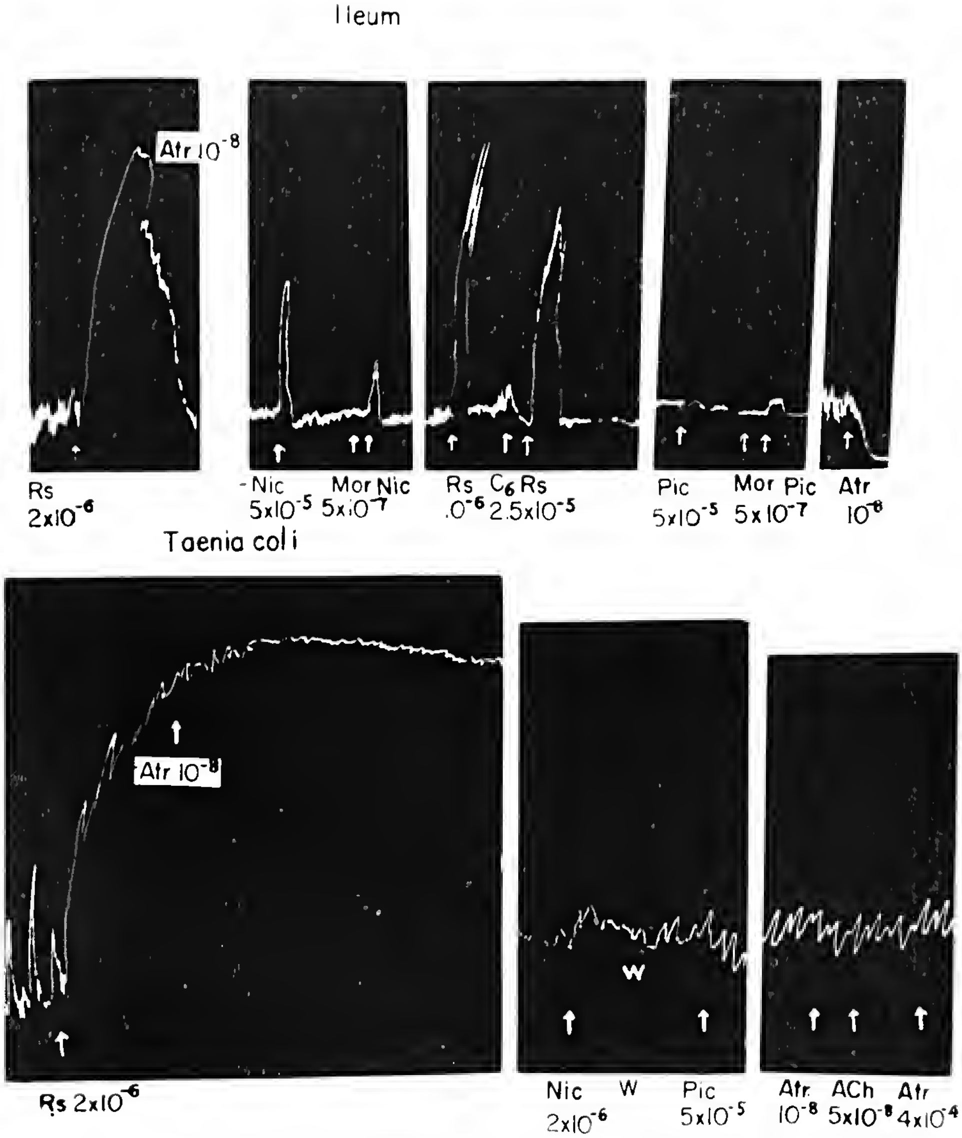
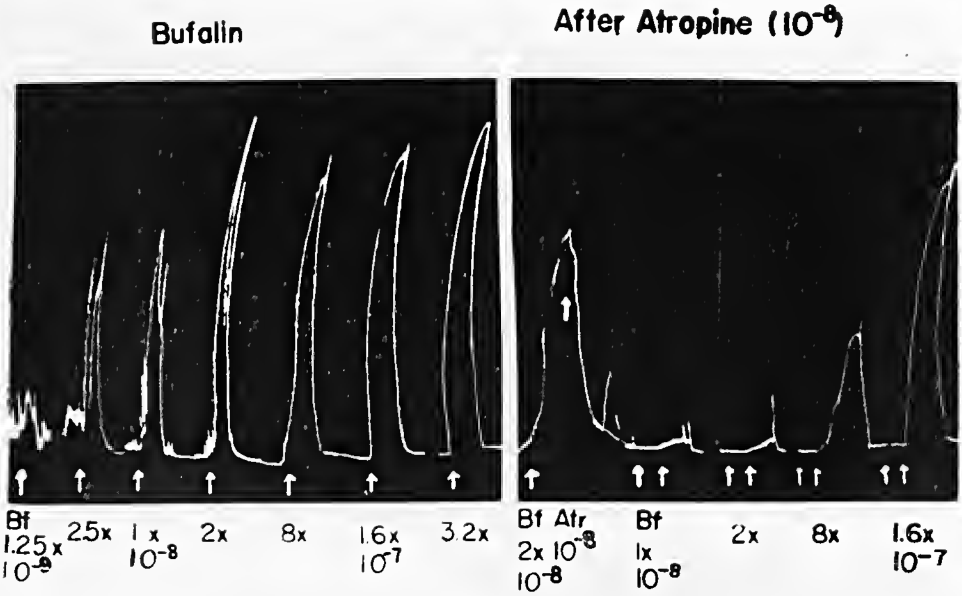


Fig. 3 — Comparison of the Effect of Resibufogenin on Ileum and Taenia coli.

Based on these results, we can assume that Ach release participates from the contracting action of bufogenins, in addition to their direct actions on the excitable membrane of the smooth muscle, as reported by H. J. Schatzmann *et al.* for the taenia-coli preparation. In the phase of relaxation there seems to be no participation of Ca^{++} release, this relaxation seems to depend upon the direct inhibitory effect exerted on the excitable membrane.

It is known that bufogenins and allied compounds, provoke a rise in blood pressure, as a consequence of an augmentation of peripheral vascular resistance resulting from vasoconstriction effects. In the spirally cut aortic strip preparation



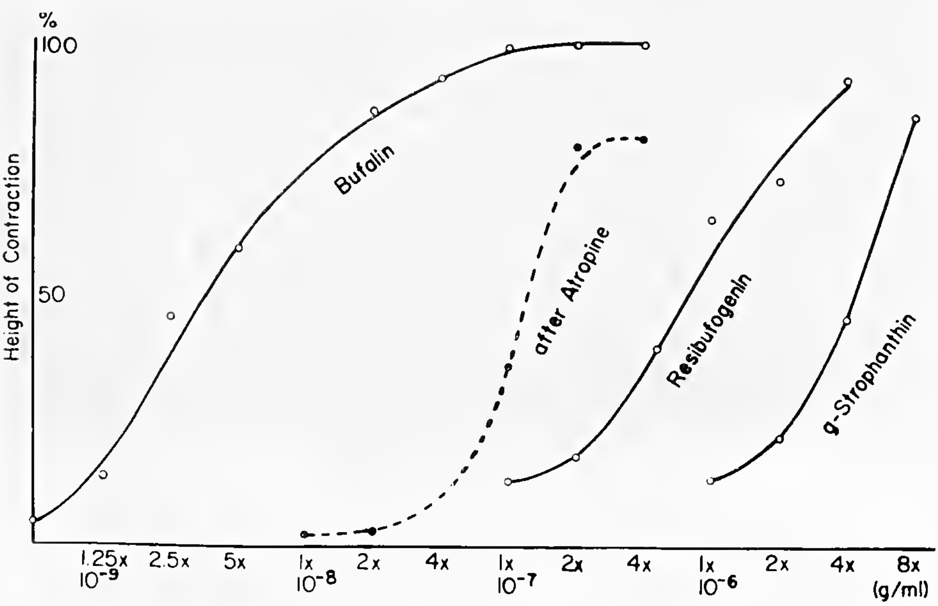


Fig. 4 — Actions of Bufogenins on the small intestine.

arranged according to G. Paterson, bufogenin did not cause such a remarkable contraction as in the ileum preparations, but only a slight rise of muscular tone. However, they potentiated the contraction induced by epinephrine and KCl. These phenomena resembles the effect of Ca^{++} on the K contracture (Fig. 6). The peak of maximum effect is in the 1.5 times concentrated Ca^{++} solution. This

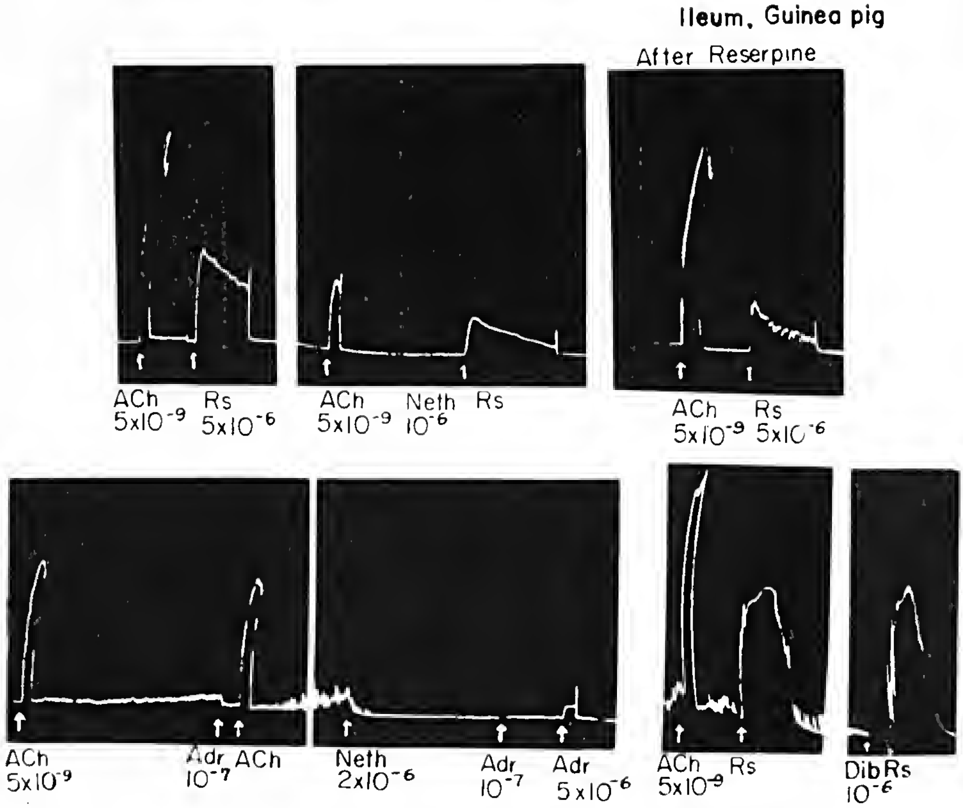


Fig. 5 — Effect of Nethalide and Dibenzyline on the intestinal contraction produced by Bufogenin.

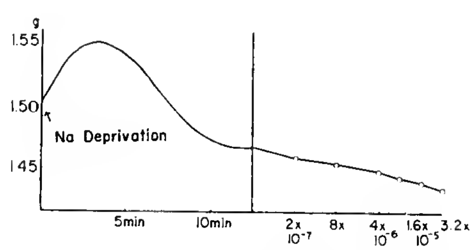


Fig. 6 — Action of Bufogenin of the Tracheal Strip immersed in Na-free Krebs Solution.

concentration of Ca^{++} augmented the contraction produced by K. Other differences, between the actions of bufogenins upon the blood vessels and heart tissue were observed and will be referred to later.

The effects of bufogenins on the tracheal smooth muscle were studied on the spirally cut tracheal strip preparation described by J. W. Constantine. In contrast to their effects on the aortic preparation, where we could not observe any

relaxation, even after the application of concentrated bufogenin solutions, in the tracheal tissue we observed relaxation following an increase in muscular tone. Occasionally we observed a few cases presenting only relaxation. In the stage of relaxation, bufogenin reduced the contractions produced by Ach and histamine (Fig. 7).

The effects of bufogenins and g-strophanthin on the guinea-pig tracheal strip preparations are shown in Fig. 8. Though relaxation following the increase

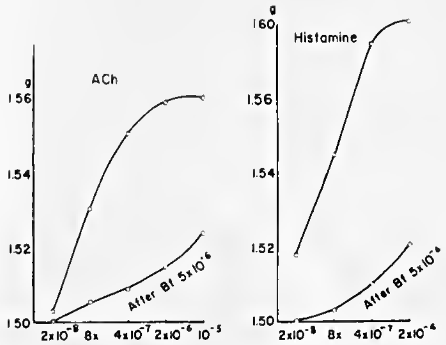


Fig. 7 — Effects of Bufogenins on the Tracheal contractions of the Guinea-pig.

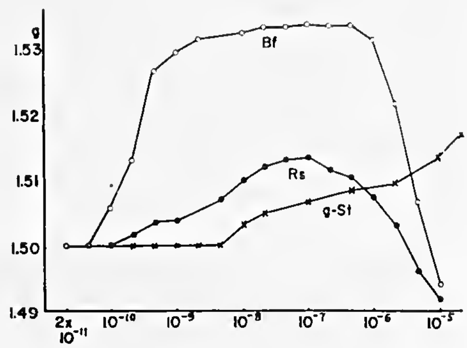


Fig. 8 — Effects of Bufogenins and Allied Compound on Guinea-pig's Tracheal Preparation.

in muscular tone are the responses induced by resibufogenin and bufalin, g-strophanthin did not cause any relaxation even after using high doses. Comparing the actions of resibufogenin and bufalin, we observed that the inhibitory effect of resibufogenin is faster in appearance in spite of its weaker contracting effect.

Studying the effects of ions upon the relaxing action, I found that the increase in muscular tone disappeared in the Na^+ free perfusion, and only the relaxing effect persisted (Fig. 9). H. J. Schatzmann supposed, based on the report of Caldwell and Keynes, that the first contracting phase of the taenia-coli preparation is dependent on the inhibition of Na^+ efflux (Na^+ pump) resulting from the action of oubain on the outer side of the muscle cells membrane, and the second relaxing phase is caused by the inhibition of Na^+ influx resulting from its action on the inner side of the muscle cells membrane after the permeation of the cells.

This hypothesis permits an explanation for the absence of the contracting phase after the perfusion of solutions Na-deprived, and I suppose that the difference of action among resibufogenin, bufalin and g-strophanthin shown in Fig. 9, depends on the difference of the permeative properties, such as shape and size of their molecules.

A comparision of the effects of bufogenins on the various smooth muscle tissues and rectus abdominis muscle of frogs, and their action on the completely depolarized membrane in isotonic K_2SO_4 Krebs solution is shown in Fig. 10.

In the guinea-pig ileum and trachea we could recognize the relaxation after the application of a high concentration of bufogenin. On the other hand, in the aortic strip, heart tissue and rectus abdominis muscle of frogs we observed only the contraction without the relaxation phase. All other tissues than the ear-

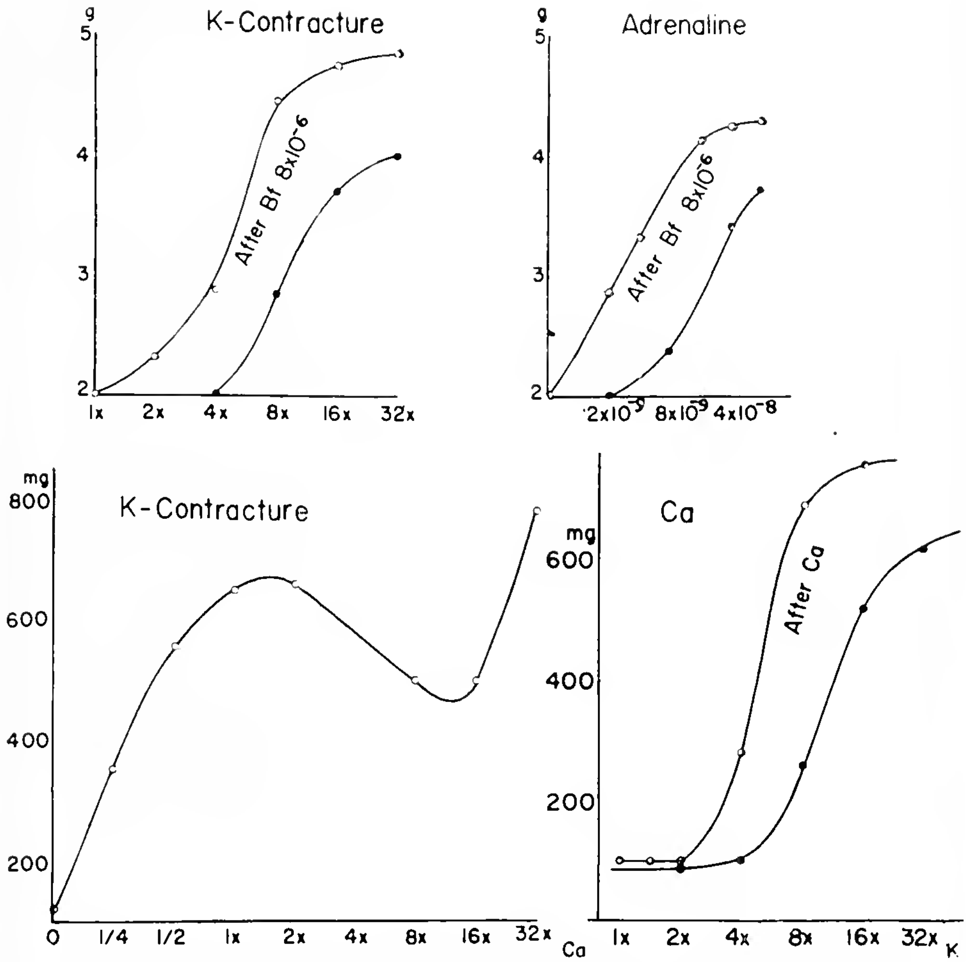


Fig. 9 — Effects of Bufogenins and Ca on the K- and Adrenaline-Contractions of Guinea-pig's and Rabbit's Arterial Strip Preparations.

diac one lost both the contracting and relaxing responses to bufogenins after K induced depolarization. Therefore, the potentiating action of cardic steroid on the smooth contraction depends chiefly on the direct action on the cell membrane, while on the cardiac tissue another mechanism participates than the direct membrane action.

The above mentioned data, regarding my investigations on the actions of bufogenins upon the various smooth muscle tissues, seem to indicate that the comparatively rapid contracting response of the ileum is due to a Ach releasing mechanism. In addition to this direct stimulating action, in the other muscle tissues examined (taenia-coli, blood vessels, trachea and frogs rectus abdominis muscle), the cell membrane seems to be chiefly involved, and there are some differences in the forms the cell membrane is directly actuated by the bufogenins.

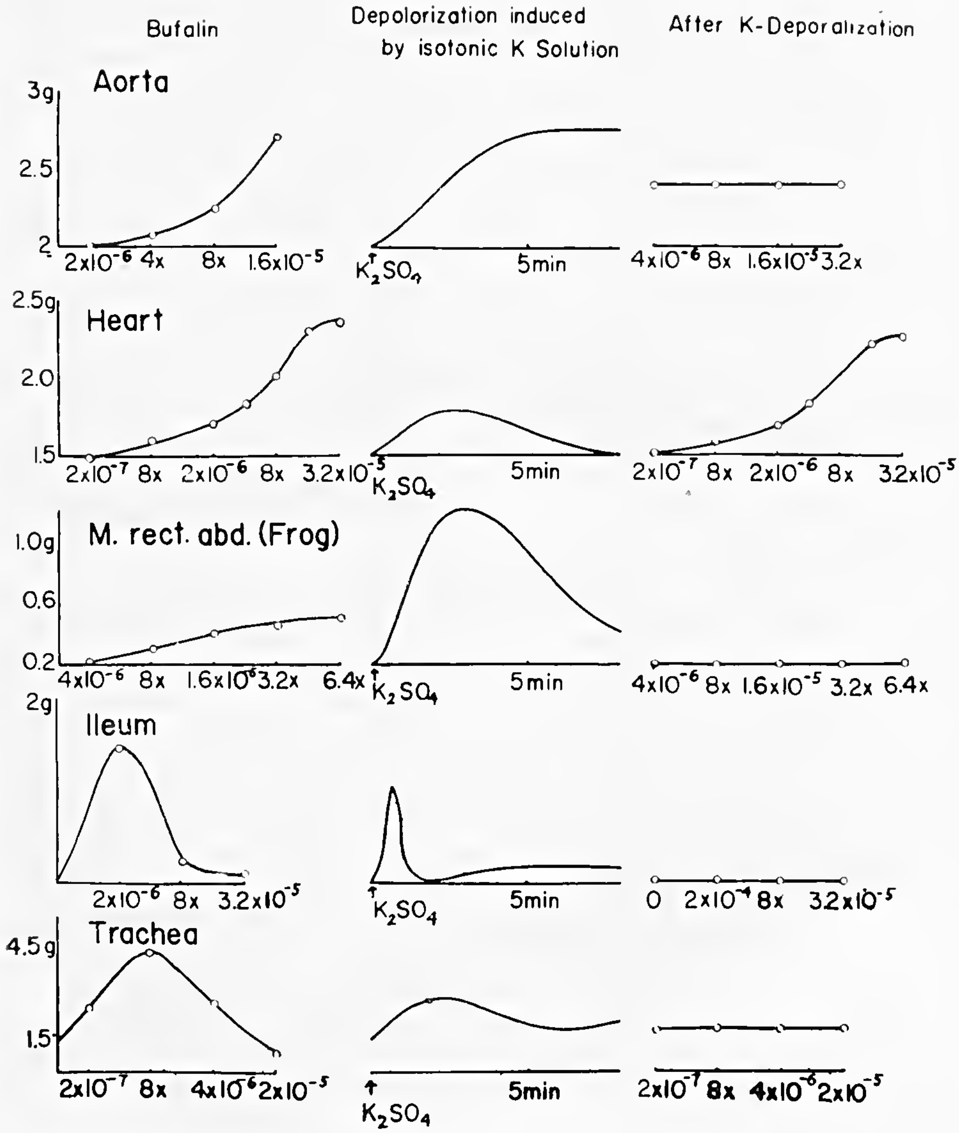


Fig. 10 — Actions of Bufogenin on the Smooth Muscle Tissues and K induced Depolarization.

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43. PHARMACOLOGY OF CRYSTALLINE CROTOXIN. I. TOXICITY

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Crotoxin is a crystallline toxin isolated for the first time in 1938 from the venom of the South American rattle-snake (*Crotalus durissus terrificus*) by Slotta and Fraenkel-Conrat (1) working at the laboratories of Butantan Institute. It behaved as a single pure protein when it was examined by E. J. Cohn's method (1), electrophoresis (2) or sedimentation and diffusion experiments in the ultracentrifuge (3). Its molecular weight was estimated to be 30.000 (3). However, as Neumann and Habermann (4, 5) have more recently shown, crotoxin can be separated into two components by column chromatography. One of these exhibits the same toxicity as crotoxin but is devoid of known enzymatic activities. It was called "crotactin" by Neumann and Habermann. The other is a phospholipase A, which according to the German authors, presents only a little toxicity. Thus crotoxin is now believed to be made up from two proteins probably united by ionic bonds.

Pharmacological data concerning crotoxin are very scarce. In fact, little more than its high toxicity for mice (6) and its stimulatory effect on the isolated guinea-pig ileum (1), was known about its pharmacological properties. Therefore, a pharmacological study of crotoxin was undertaken and will be presented in this and in the following communications.

Material and methods

The venom used in this research came from a serpentarium at Catalão, Goiás. It was a dried pool of *C. d. terrificus* venom devoid of crotamine.

Both methods devised by Slotta and Fraenkel-Conrat (1) for separating crotoxin were utilized. Nearly all batches of it were obtained in crystalline form. Crotoxin, as well as the venom was dissolved in saline immediatly before use. However, in some experiments a solution kept in the refrigerator at 4°C for no more than 7 days was also employed.

The median lethal doses of crotoxin were determined in mice, rats, pigeons, guinea-pigs and rabbits and those of the venom, in mice and rats. Four dose levels spaced in a geometric progression (common ratio 1.5) were employed. Six animals per dose were used, except in the determination of the LD_{50} for pigeons. In this case, three animals per dose were employed.

This study was supported by a grant from the "Fundação de Amparo à Pesquisa do Estado de São Paulo".

The intravenous route was used for the determination of the median lethal doses of crotoxin and the venom for mice (17-21 g). In addition, crotoxin median lethal dose by subcutaneous route for mice was also determined. An "Agla" micrometer syringe outfit was used in this case to inject the crotoxin solution. In other determinations, crotoxin or the venom was administered by intravenous, intraperitoneal or intramuscular route. Pigeons, guinea-pigs, mice and rats were observed for 48 hours. The rabbits were discarded after the fifth day. The median lethal doses and their 95 of 100 confidence intervals were calculated by the procedure described by Weil (7).

The signs evoked by crotoxin in dogs were investigated in 36 conscious animals. The intravenous route was employed. The animals were observed for 5 to 9 days. Ten dogs, however, were anaesthetized 24 or 48 hours after crotoxin administration in order to prepare them for the study of neuromuscular transmission. To some of these animals artificial respiration was given after their spontaneous respiration had ceased. An automatic Takaoka respirator in connection with an air or oxygen cylinder was used. The urine of nearly all dogs was examined for albumin and haemoglobin. Diuresis was studied in 13 female animals which were kept for this purpose in metabolic cages for 5 or more days after crotoxin injection. In most instances, controls were performed by collecting the urines during the four days that preceded crotoxin administration.

Sixteen of the dogs were used for the determination of the crotoxin median paralysing dose. Four dose levels in a geometric progression with a common factor of 1.25 were employed. The ED_{50} was also calculated according to Weil procedure. Three unanaesthetized dogs were also intravenously injected with the venom.

The motor effects evoked by crotoxin and gallamine (Flaxedil) were compared in monkeys by using two *Cebus sp.*. Both substances were intravenously administered.

Conscious cats were injected with crotoxin by intravenous and intra (cerebro) ventricular routes. Three animals were used in both instances. Crotoxin was injected in one of the brain lateral ventricules through a Feldberg cannula inserted some days before.

Results

Crotoxin showed itself to be more toxic than the venom for both mice and rats. The ratio of their median lethal doses for these animals did not differ significantly (Table I).

Great species variability was found in the sensitiveness to crotoxin. Pigeons proved to be extremely sensitive while rats were very resistent to its lethal action (Table I). Crotoxin mean lethal dose by subcutaneous route for mice was 2.16 (1.15 to 4.02) times greater than that determined by intravenous administration. The crotoxin median paralysing dose for dogs was estimated to be 138 (106 to 179) mcg/Kg. Its mean lethal dose for this animal species was probably greater than 200 mcg/Kg (Table II).

Crotoxin given by the usual parenteral routes of administration elicited paralysis in all animal species in which it was injected. In nearly all (rats, guinea-pigs, rabbits, cats, dogs and monkeys) of them, the motor effects which began by muscular hypotonia and muscular weakness and evolved to flaccid paralysis, were identical with those observed in animals injected whith curare. The order of involvement of the various muscular groups was also the same in both

TABLE I — CROTOXIN AND *CROTALUS DURISSUS TERRIFICUS* VENOM MEDIAN LETHAL DOSES AND THEIR 95 OF 100 INTERVALS FOR VARIOUS ANIMAL SPECIES

Animal	Route of administration	Crotoxin LD_{50} (mcg/Kg)	Venom LD_{50} (mcg/Kg)	Venom LD_{50}/ Crotoxin LD_{50}
Pigeon	Intravenous	2.17 (1.35 to 3.48)	—	—
Guinea-pig	Intramuscular	38 (29 to 50)	—	—
Mouse	Intravenous	82 (66.9 to 101.4)	168.5 (135.7 to 209.85)	2.05 (1.34 to 3.14)
	Subcutaneous	177.5 (117 to 269)	—	—
Rabbit	Intravenous	110.23 (85.64 to 141.9)	—	—
Rat	Intraperitoneal	756 (564 to 1010)	1950 (1950 to 2120)	2.58 (1.77 to 3.75)

cases. The similarity of signs evoked by crotoxin and curare was very convincingly shown in monkeys (Fig. 1). Pigeons and mice were exceptions. In these animals, only large doses produced the curare-like paralysis. Small doses elicited an ascendent type of paralysis. Mice, for instance, injected with a dose of crotoxin near its LD_{50} frequently showed after a long delay, paralysis of their hind limbs while the neck and fore limb muscles were not affected.

Fig. 1 — From left to right: (1) a monkey 15 hours after receiving 100 mcg/Kg of crotoxin i.v., (2) the same monkey 30 hours later after nearly complete recovery and (3) another *Cebus sp* 3 to 5 min. after being intravenously injected with 300 mcg/Kg of gallamine (Flaxedil). Ptosis of the eyelids and of the jaw, paralysis of the muscles of the neck as well as a generalized loss of muscle strength occurred in both monkeys. They also became aphonic, could not swallow and presented diaphragmatic respiration. Consciousness in both animals seemed to be preserved.

The diaphragm was one of the last muscles to become paralysed in crotoxin intoxication. When it ceased contracting, the animals could be maintained alive under artificial respiration. This was demonstrated in cats and dogs. Crotoxin paralysis proved to be reversible in all animals except rabbits. These animals always died before recovering from the paralysis even when it was not complete.

The first signs of the motor effects caused by not too large a dose of crotoxin appeared after a long delay and the paralysis lasted for long periods of time. In dogs, for instance , the onset of loss of muscle strength generally occurred from 2 to 4 hours after the intravenous injection of 160 to 250 mcg/Kg of crotoxin and the animals became paralysed for 48, sometimes 72 hours, or even more. However, the lag between crotoxin administration and the onset of paralysis was usually a little shorter in the small laboratory animals. In addition, the duration of paralysis in the animals did not last so long.

During crotoxin paralysis consciousness and sensitivity seemed to be preserved. Paralytic dogs, for instance, shook their tails when the observer whistled or tickled them, or made efforts to move when their skin was pinched. Preservation of consciousness was also testified by the reaction that a paralytic dog presented at the incidental approach of a cat. It became furious making desperate efforts to fight with the cat.

TABLE II — SOME EFFECTS OF CROTOXIN IN DOGS

Effects	Doses of crotoxin (mcg/Kg)			
	200	160	128	102.4
Defaecation	2/4	3/4	2/4	1/4
Vomiting	3/4	2/4	0/4	1/4
Salivation	2/4	1/4	0/4	0/4
Slight muscular hypotonia (1)	4/4	4/4	4/4	4/4
Advanced muscular hypotonia and considerable loss of muscle strength (2)	4/4	2/4	3/4	2/4
Flaccid paralysis(3)	4/4	2/4	2/4	1/4
Death	1/4	0/4	1/4	0/4

(1) Some incoordination and weakness in muscular action were perceivable when the dogs were forced into locomotion.

(2) The dogs could not support themselves on their limbs because of the loss of muscular strength.

(3) The dogs lay dow helpless on one of their flanks.

Besides paralysis, other effects were observed in unanaesthetized dogs. Prior to the appearance of paralysis, one or more of the following effects frequently occurred: defaecation, vomiting, salivation (Table II). During the paralysis and after its partial or complete regression, or even in its absence, albuminuria, haemoglobinuria and oliguria or anuria (Table III) were verified. Opacity and exfoliation of the cornea as well as vomiting occurred in a few dogs after the third day of crotoxin administration. Vomiting and salivation were also observed in cats before the onset of paralysis.

TABLE III — EFFECT OF CROTOXIN ON DIURESIS

Dog (♀) Weight (Kg)	Crotoxin Dose (mcg/Kg)	Diuresis: Before crotoxin administration Mean of 4 days (ml/day)	Diuresis: After crotoxin administration Mean of 5 days (ml/day)	Diuresis: Per cent reduction
11.0	250	—	61.0	—
5.7	250	—	36.0 *	—
5.7	250	—	49.0	—
8.1	250	—	53.0	—
6.2	250	183.0	62.0	66.1
6.7	160	—	94.0	—
7.9	160	149.0	200.0	Increased
6.4	160	212.5	109.0	48.7
7.9	160	312 5	168.0	46.2
6.1	128	200.0	167.0	16.5
5.9	128	337.5	52.0 **	84.5
4.9	128	140.0	105.0	25.0
6.5	102.4	185.0	84.0	54.6

* Mean of 2 days.
** Mean of 4 days.

Deaths of the dogs generally took place within the first 48 hours of crotoxin injection. They were due to respiratory arrest. However, a few animals died afterwards when the paralysis was in regression. There was post-mortem evidences that in these cases shock was the cause of death. Renal lesions were demonstrated in the kidney of nearly all dogs injected with crotoxin. They will be reported in a separate communication. Hemorragic foci were never seen in the meninges or brain substance of the dogs killed by crotoxin action.

When crotoxin (50 mcg) was injected by intra (cerebro) ventricular route in cats (1 to 5.5 Kg) seizures of clonic convulsions ending in death were produced 2 to 3 hours after its administration (Fig. 2). Tachypnoea, slight clonic movements in the paws, salivation and vomiting were observed prior to the convulsive seizures.

The venom evoked signs in mice which were similar to those produced by crotoxin. In rats and dogs, however, the symptomatology differed from that elicited by crotoxin, chiefly in the latter species.

Rats injected with venom became very flaccid but never showed complete paralysis as the rats under action of crotoxin. Respiratory arrest seemed to take place before the motor effects attained that point.

The venom (250 mcg/Kg. i.v.) elicited the following effects in dogs:

Fig. 2 — This cat (1.3 Kg) was injected with 50 mcg of crotoxin by intra (cerebro) ventricular route. The seizures of clonic convulsions (fotograph) appeared 2 hours and 20 minutes after crotoxin administration. Tachypnoea, salivation and vomiting occurred prior to the convulsive fits.

1. Immediately after its injection, tachypnoea, defaecation, micturition as well as a very short period of equilibrium loss, and nystagmus were observed. A short time later defaecation, vomiting and salivation occurred.

2. After a delay of 1 or more hours, seizures of tonic-clonic convulsions ending in death or clonic convulsive movements, usually of the limbs associated with some degree of muscular hypotonia, and loss of muscular strength happened.

All three dogs died: one of them 2 hours after the injection of the venom, another between the first and the second day and a third dog in from the third to the fourth day. Post-mortem, this last animal showed small haemorragic foci in the pia mater and zones of congestion and oedema in the lungs, besides intensily congested abdominal viscera. The mucosa of its intestines was congested and haemorragic.

Discussion

The results obtained in this research point out the following order of susceptibility to the lethal action of crotoxin: pigeons > guinea-pigs > mice > rabbits > rats (see Table I). It seems to be important, therefore, to inquire if this same order of sensitiveness occurs in relation to the venom. Our results showed

that the venom was also much more toxic for mice than for rats. Brazil and Pestana (8), on the other hand, encountered the following values for the minimal lethal doses of the venom: 1 mcg (3.3 mcg/Kg, i.v.) for pigeons, 60 mcg/Kg (i.m.) for guinea-pigs and 250 mcg/Kg (i.v.) for rabbits. It can be inferred, therefore, from these results that the order of susceptibility to the venom among pigeons, guinea-pigs, mice, rabbits and rats, is the same as that shown to crotoxin. The venom was found to be less toxic than crotoxin for mice and rats (see Table I). The figures given by Brazil and Pestana (8) refer to the minimal lethal doses of the venom and therefore are not strictly comparable with the values of the median lethal doses we determined for crotoxin. Nevertheless the analysis of their data suggests that the venom is also less toxic than crotoxin for pigeons, guinea-pigs and rabbits. As, in addition, the signs evoked by small doses of the venom and crotoxin in these animals are very close or even indistinguishable, it can be concluded that crotoxin is the component which produces the signs of envenomation and causes death in pigeons, guinea-pigs, mice and rabbits when small doses of the venom are injected. In rats which are very resistant to crotoxin (and also to the venom), other venom components participate in the envenomation, altering the symptomatology elicited by crotoxin. For dogs, the venom seems to be as toxic as crotoxin and, in addition, evokes effects, when given by intravenous route, which are very different from those produced by that substance. It gives rise, for instance, to convulsions and, sometimes, to small haemorragic foci in the pia, as first pointed out by Brazil (8, 9) and confirmed in this study. The convulsive action of the venom could be due: (a) to the penetration of crotoxin in the brain interstitial spaces after the breakdown of the blood-brain barrier produced by another component of the venom, or (b) to a toxin of the venom different from crotoxin. The first hypothesis is suggested by the convulsive action of crotoxin when introduced by intra (cerebro) ventricular route. The second one is based on the separation of a fraction from the venom which caused convulsions not only in dogs but in mice as well (unpublished data).

Several signs of systemic envenomation produced in man by the bite of the South American rattle-snake were observed in the animals injected with crotoxin, specially monkeys and dogs. Such were ptosis of the eye-lids and of the jaw, weakness of the neck and other skeletal muscles as well as general muscle paralysis. Difficulty in swallowing occurred in dogs and monkeys and is a complaint of some patients. Ptosis of the eye-lids is one of the first signs of envenomation and is probably displayed by nearly all patients bitten by *C. d. terrificus*. Vomiting within the first hours of the bite also occurs and was frequently observed in dogs injected with crotoxin (Table II). Albuminuria, haemoglobinuria, oliguria and even anuria, which were effects produced by crotoxin in dogs, are frequently observed in patients (10). However, participation of other venom components in the genesis of renal disturbances and vomiting is not excluded.

Summary

1. The toxicity of crotoxin was compared with that of the venom of *Crotalus durissus terrificus* by determining their median lethal doses for mice (i.v.) and rats (i.p.). Crotoxin showed itself to be more toxic than the venom for both animal species.

2. The median lethal doses of crotoxin for pigeons (i.v.), guinea-pigs (i.m.) and rabbits (i.v.) were also determined. The following order of susceptibility to

its lethal action was encountered: pigeons > guinea-pigs > mice > rabbits > rats. It seems that the order of sensitiveness to the venom is the same.

3. Crotoxin evoked paralysis in all animal species used. In nearly all of them the paralysis was similar to that elicited by curare in the degree of flaccidity shown by the skeletal muscles and in the order of involvement of the various muscular groups. Artificial respiration maintained the animals alive after spontaneous respiration had ceased. Consciousness and sensitivity seemed to be preserved in animals presenting crotoxin paralysis.

4. Besides paralysis, defaecation, vomiting, salivation, albuminuria, haemoglobinuria, oliguria and in some instances opacity and exfoliation of the cornea, were observed in dogs.

5. When administered by intra (cerebro) ventricular route in cats, crotoxin elicited convulsions instead of paralysis.

6. There are evidences indicating that the lethal effect of small doses of the venom in pigeons, guinea-pigs, mice, rabbits and rats is due to crotoxin. In dogs, however, it seems that death is produced by a component of the venom different from crotoxin.

7. In human beings several signs observed after the bite of *C. d. terrificus* can be assigned to crotoxin action.

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41. PHARMACOLOGY OF CRYSTALLINE CROTOXIN. II. NEUROMUSCULAR BLOCKING ACTION

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In the precedent communication (1), it was shown that the motor signs observed in animals injected with crotoxin are very close to those evoked by curare. This suggested a peripheral origin for the paralysis caused by crotoxin and incited us to investigate neuromuscular transmission in animals to which this substance was administered. In the present paper, evidence is given indicating that block of neuromuscular transmission is really the cause of the paralysis. Some characteristics of the neuromuscular blockade elicited by crotoxin was also investigated as well as ganglionic transmission in animals injected with this venon component. In addition the effect it produces on the sensitiveness of striated muscle to acetylcholine or potassium was studied on the isolated and cronically denervated rat hemi-diaphragm.

Material and methods

In order to decide if neuromuscular transmission is blocked or not in the animals showing the paralysis evoked by crotoxin, 10 conscious dogs were intravenously injected with 0.2 to 0.5 mg/Kg of this substance. Twenty-four or forty-eight hours thereafter, they were anaesthetized with sodium pentobarbital (20 mg/Kg, i.v.) and prepared as follows. A drill was inserted in the superior third of the tibia of one of the legs and attached through a special adjustable block to a upright fitted on a iron base plate in order to immobilize the limb of the animal. The peroneal nerve was exposed in the upper antero-lateral aspect of the leg and sectioned. To its distal end platinum electrodes were attached. The tendon of the tibialis anterior muscle was then exposed, sectioned, and connected by means of a strong thread to a flat spring steel myograph of a Brown-Schuster myograph apparatus. Two needle electrodes were inserted in the muscle, one in its belly, the other near the tendon. The trachea was exposed and cannulated. A tracheal cannula provided with two upper lateral outlets was used. In most experiments one of these was put in connection with a Marey tambour in order to record the respiratory movements of the animal. A François Franck cannula was then inserted in one of the carotids for recording the arterial blood pressure through a Ludwig's manometer. A polyethylene cannula was inserted in the femoral vein of the opposite side to the prepared limb for intravenous administration of drugs.

This investigation was supported by a grant from the "Fundação de Amparo à Pesquisa do Estado de São Paulo.

Nerve stimulation was carried out with supramaximal shocks of 0.2 msec duration delivered at a rate of 30 per minute. When the muscle did not respond to stimuli of up to 100 v. applied to the nerve, it was assumed that transmission was completely blocked. Stimuli of a higher (50 c/s) frequency were also employed for short (10 to 40 seconds) periods of stimulation. Direct muscle stimulation was made with shocks of 2 msec. duration and 50 to 100 v. The stimuli used were square wave pulses from a Grass S 4 Stimulator.

Neostigmine methylsulfate (0.1 mg/Kg) and calcium chloride (0.5 ml/Kg of a 10% solution, as well as a 10% solution of sodium bicarbonate, were intravenously injected in some dogs.

The neuromuscular blocking action of crotoxin was also studied in cats. Sixteen animals were anaesthetized with sodium pentobarbital (30 mg/Kg, i.v.) and prepared as described for dogs. Nerve stimulation was made with supramaximal pulses of 0.2 msec duration delivered at a rate of 6 per minute. Shocks of 2 msec duration and 50 to 100 v. were used for directly stimulating the muscle. Large doses (0.5 mg/Kg and 1 mg/Kg, i.v.) of crotoxin were used in order to obtain neuromuscular blockade within the first three hours of its administration. The effect produced by neostigmine methylsulfate (0.1 mg/Kg, i.v.), edrophonium chloride (0.5 mg/Kg, i.v.) and succinylcholine chloride (0.02 or 0.05 mg/Kg, i.v.) on the neuromuscular blockade was investigated.

Ganglionic transmission was studied in four of the cats which were utilized in the investigation of the neuromuscular action of crotoxin. The vagus-sympathetic trunk was exposed and sectioned. In two cats, the sympathetic chain was, in addition, separated from the vagus nerve. Platinum electrodes were then attached to the upper end of either the sympathetic chain or the whole vagus-sympathetic trunk. The head of the animal was firmly fixed by a cat holder and the nictitating membrane connected with a Starling heart lever. Pulses of 0.2 msec duration and 2 to 5 v. delivered at a rate of 5 per second were used. Arterial blood pressure was also recorded.

Artificial respiration was employed whenever necessary.

Crotoxin-acetylcholine and crotoxin-potassium antagonism were investigated on the isolated and cronically denervated rat hemidiaphragm preparation. The left phrenic nerve of rats was sectioned under ether anaesthesia as described in a previous paper (2). After 25 to 35 days, the rats were anaesthetized with chloral hydrate (300 mg/Kg, i.p.) and bled. The denervated hemi-diaphragm was then removed and suspended in a organ bath containing 8 ml of Tyrode solution with 0.2 per cent glucose at 37°C. The preparation was aerated with a mixture of 95% O_2 and 5% CO_2. The contractures were recorded with an isotonic lever. Logarithmically spaced doses (common ratio 2) of acetylcholine or potassium chloride were added to the bath and allowed to act for 30 to 40 seconds. After each addition of acetylcholine or potassium chloride the bath fluid was changed four times and the preparation left for 10 minutes. The following doses of crotoxin were used in the experiments with acetylcholine: 0.5×10^{-6}, 10^{-6}, 2×10^{-6} 4×10^{-6}. In the experiments with potassium, only one dose (2×10^{-6}) of crotoxin was employed. All contractures were expressed as percentages of the contracture obtained by immersing the preparations in a 0.1M K_2SO_4 solution.

The crotoxin used in this investigation was prepared as indicated in the precedent paper (1).

Results

Block of neuromuscular transmission was demonstrated in all of the 10 dogs which had been injected 24 or 48 hours before the experiment with crotoxin (Table I). The blockade was complete in 4 of them and partial (Fig. 1) in the remaining. Neostigmine, calcium chloride and sodium bicarbonate did not remove the neuromuscular blockade nor ameliorate the transmission. A partial recovery of muscle responses or a great increase of them always appeared after

TABLE I — NEUROMUSCULAR TRANSMISSION * AND ARTERIAL BLOOD PRESSURE OF DOGS 24 OR 48 HOURS AFTER INTRAVENOUS INJECTION OF CROTOXIN

Crotoxin dose (mcg/Kg)	Hours after injection	Neuromuscular blockade	Arterial blood pressure (mm Hg)
500	24	complete	170
500	24	partial	120
500	48	partial	116
250	24	complete	104
250	24	partial	220
250	24	complete	84
250	48	partial	—
250	24	complete	230
200	24	partial	186
200	24	partial	160

* Sciatic nerve-tibialis anterior muscle preparation.

tetanic stimuli were applied to the nerve (post-tetanic facilitation, Fig. 1). Cessation of the spontaneous respiration occurred in three dogs in early stages of the experiments; artificial respiration secured their survive. All dogs showed flaccid paralysis and their respiration was diaphragmatic before being anaesthetized.

In the cat experiments, crotoxin produced complete or partial neuromuscular blockade within the first 3 hours of its administration. Inhibition was complete at that time in 3 out of the 9 animals injected with 0.5 mg/Kg and in 5 out of the 7 to which 1 mg/Kg of crotoxin was administered. Neostigmine was without effect on the neuromuscular block. Edrophonium increased the partially inhibited responses of the tibialis anterior muscle. However, the effect was short-lived (Fig. 2). Succinylcholine also increased the twitches in 6 out of 8 experiments (Fig. 3). This effect was produced by doses of the depolarizing drug which caused partial blockade before crotoxin administration (Fig. 3).

The stimulation of the cervical sympathetic trunk elicited contractions of the nictitating membrane in all four cats. In two animals, the contractions attained a very good tension (Fig. 4). These cats had been injected with 0.5

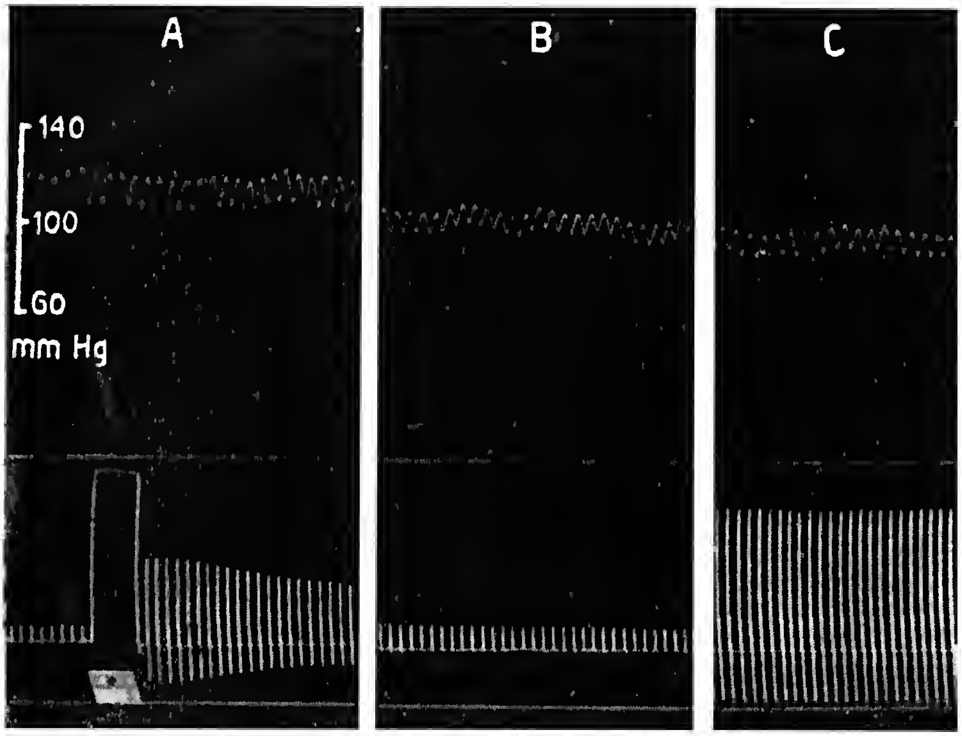

Fig. 1 — Dog, 7 Kg, sodium pentobarbital. Records of arterial blood pressure and contractions of tibialis anterior muscle. The peroneal nerve was stimulated at A and B with supramaximal shocks of 0.2 msec duration delivered at a rate of 30 per minute; at signals, shocks of a high (50 cycles/second) frequency were applied to the nerve for 10 seconds. At C, direct stimulation (pulses of 100 v. and 2 msec duration delivered at a rate of 30 per minute) of the tibialis anterior muscle. This animal had been intravenously injected 48 hours before the experiment with 0.25 mg/Kg of crotoxin. It was paralytic when anesthetized with 20 mg/Kg of sodium pentobarbital to be prepared for recording arterial blood pressure and the contractions of the tibialis anterior muscle.

mg/Kg of crotoxin. In the other two, the contractions were somewhat weaker but had no tendency to decrease in tension during the experiments. Therefore, it seems that there was also no ganglionic blockade in these animals which had been injected with 1 mg/Kg of crotoxin. In all four cats, the responses of the tibialis anterior muscle were already blocked and in three of them, the spontaneous respiration, in addition, had ceased before the beginning of the experiments.

Crotoxin produced a shift of the dose-response curve for acetylcholine to the right. A slight decline of the slopes of the curves occurred (Fig. 5). The inhibitory effect produced by crotoxin on the acetylcholine contractures could not be removed by washing repeatedly the preparation. However, some decrease of it was obtained. The dose-response curve for potassium (Fig. 6) was slightly shifted to the left by 2×10^{-6} of crotoxin. The contractures elicited by acetylcholine or potassium after treatment of the muscle with crotoxin were not so sustained as before.

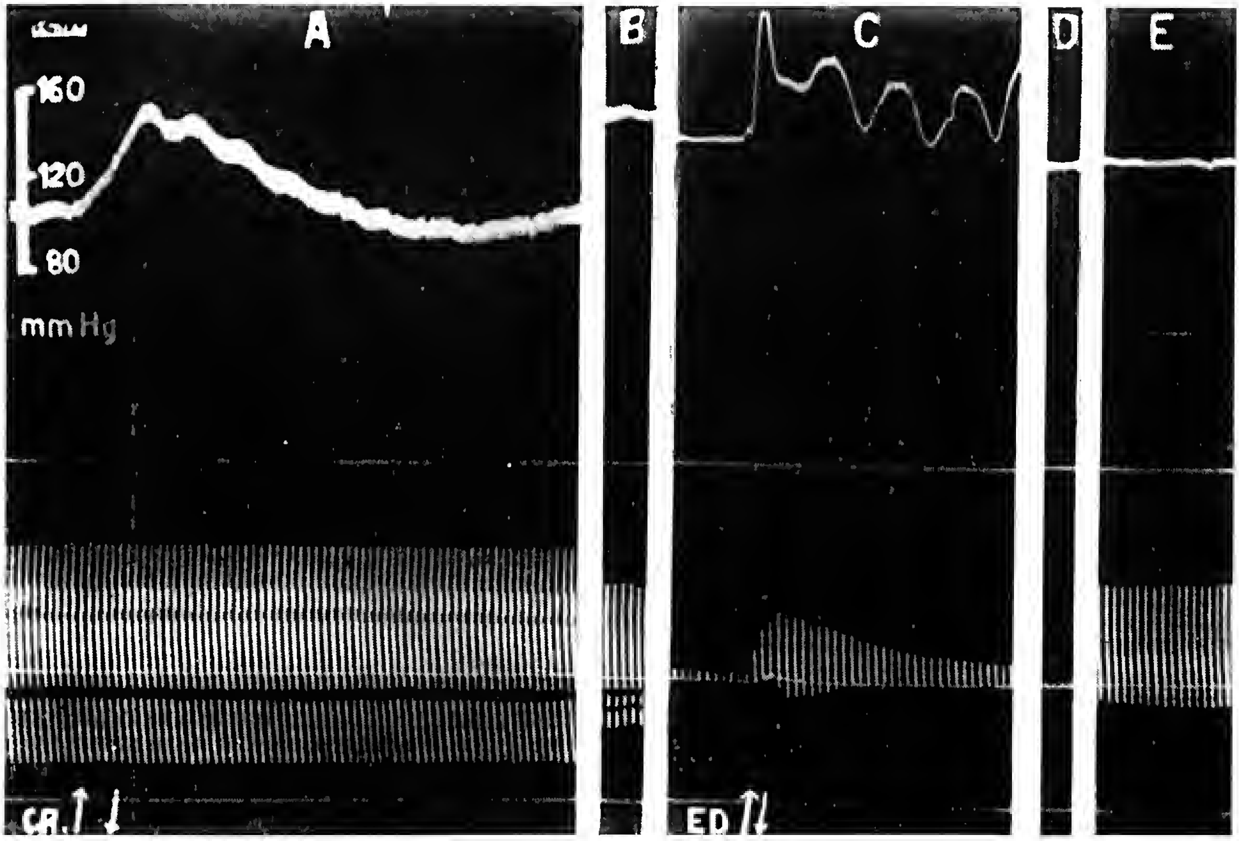

Fig. 2 — Cat, 4 Kg, sodium pentobarbital. Records of arterial blood pressure and contractions of the tibialis anterior muscle. The peroneal nerve was continuously stimulated with supramaximal shocks of 0.2 msec duration delivered at a rate of 6 per minute. At E, direct stimulation (shocks of 100 v., 2 msec timed 10 seconds apart) of the muscle was done. At Cr., 1 mg/Kg of crotoxin and at ED, 0.5 mg/Kg of edrophonium were intravenously injected. The records at B, C, D, and E were taken 70, 105, 135 and 150 minutes after A.

Discussion

This study showed that the neuromuscular blockade produced by crotoxin can per se explain the paralysis observed in the animals injected with it. This does not mean, of course, that an action of crotoxin on the central nervous system does not contribute to the paralytic effect. However, the following results do not seen to support this hypothesis. First, the experiments concerning the effects evoked by the intra (cerebro) ventricular administration of crotoxin (1) pointed out that this substance exerts a stimulating action, not a depressing one, if it gains access to the brain interstitial fluid. Secondly, consciousness and sensibility seemed to be preserved in the animals showing the paralysis evoked by crotoxin. Thirdly, the order of involvement of the various muscular groups during crotoxin intoxication was similar to that elicited by drugs which act peripherally, and differed, therefore, from that produced by compounds like mephenesin whose loci of action are in the central nervous system. Therefore,

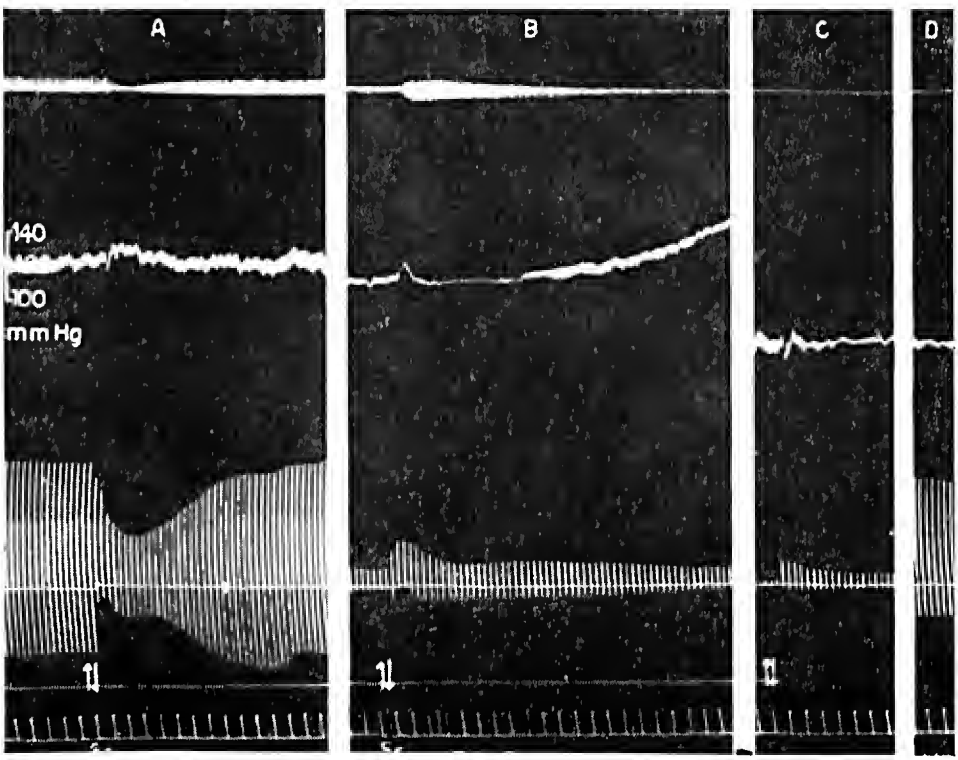

Fig. 3 — Cat, 2.4 Kg, sodium pentobarbital. Records of respiration, arterial blood pressure and the contractions of the tibialis anterior muscle. The peroneal nerve was continuously stimulated with supramaximal shocks of 0.2 msec duration delivered at a rate of 6 per minute. Direct stimulation of the muscle (at D) was done with pulses of 100 v., 2 msec timed 10 seconds apart, after complete neuromuscular block had occurred. At A, 0.05 mg/Kg of succinylcholine was intravenously injected before crotoxin administration; at B and C, the same dose of the depolarizing drug was injected 105 and 122 minutes after the intravenous administration of 1 mg/Kg of crotoxin. Artificial respiration (at C and D) was given after the spontaneous respiration had ceased.

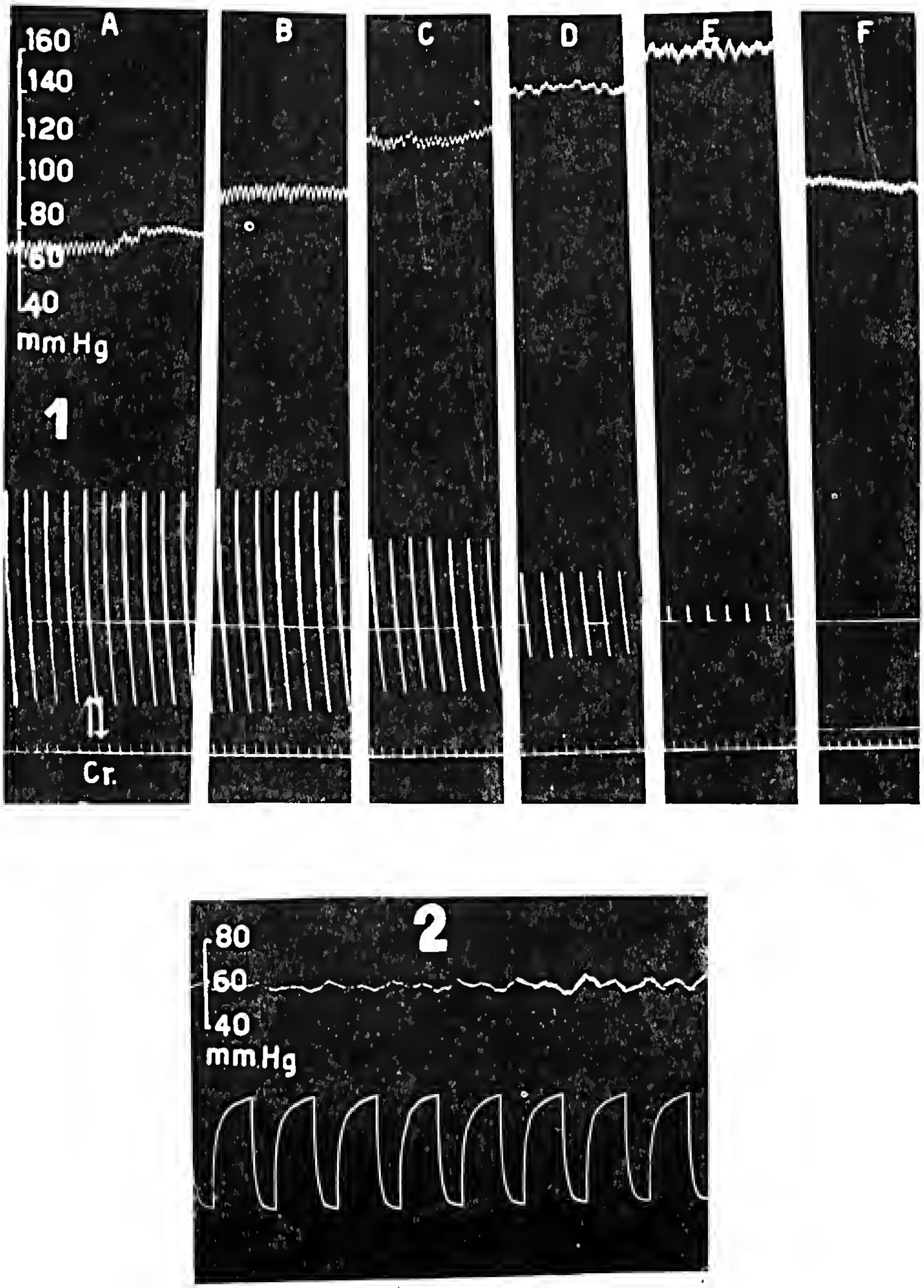

Fig. 4 — Cat, 2.5 Kg, sodium pentobarbital. 1. Records of arterial blood pressure and contractions of the tibialis anterior muscle. The peroneal nerve was stimulated with supramaximal shocks delivered at a rate of 6 per minute. B, C, D, E and F are records taken 30, 60, 90, 120 and 150 minutes after the intravenous administration of 0.5 mg/Kg of crotoxin. 2. Records of arterial blood pressure and contractions of the nictitating membrane. The cervical sympathetic chain was stimulated with shocks of 2v, 0.2 msec, delivered at a rate of 5 per second; the stimulations were done for 15 seconds periods followed by periods of 1 second rest. The records of the contractions of the nictitating membrane were taken nearly 4 hours after the administration of crotoxin.

it can be concluded that in all probability the paralysis caused by crotoxin is exclusively due to an interruption of neuromuscular transmission.

The blockade produced by crotoxin is of the non-depolarization type as can be inferred from a comparison of the results obtained in this research with those given by the study of the depolarizing drugs (3, 4). Edrophonium and succinylcholine, for instance, did not deepen the blockade as they would do if it was caused by a persistent depolarization of the end-plate region of the muscle fibres. Instead, both drugs increased although only to a small degree the partially blocked response of the cat tibialis anterior muscle. Furthermore, during crotoxin neuromuscular block, tetanization of the motor nerve was followed by a post-tetanic partial relief of it, a resnlt which also would not have occurred if transmission was interrupted by a depolarization blockade. The same conclusion can be drawn from the absence of muscle fasciculations in cats and other animals injected with crotoxin as well as from the lack of any stimulating action of this substance on the denervated rat hemi-diaphragm. Depolarizing drugs in birds give rise to contracture of skeletal muscles, not to flaccid paralysis. Small doses of crotoxin did not elicited, as previously reported(1), a flaccid paralysis of the curare type in pigeons. However, the symptoms presented by

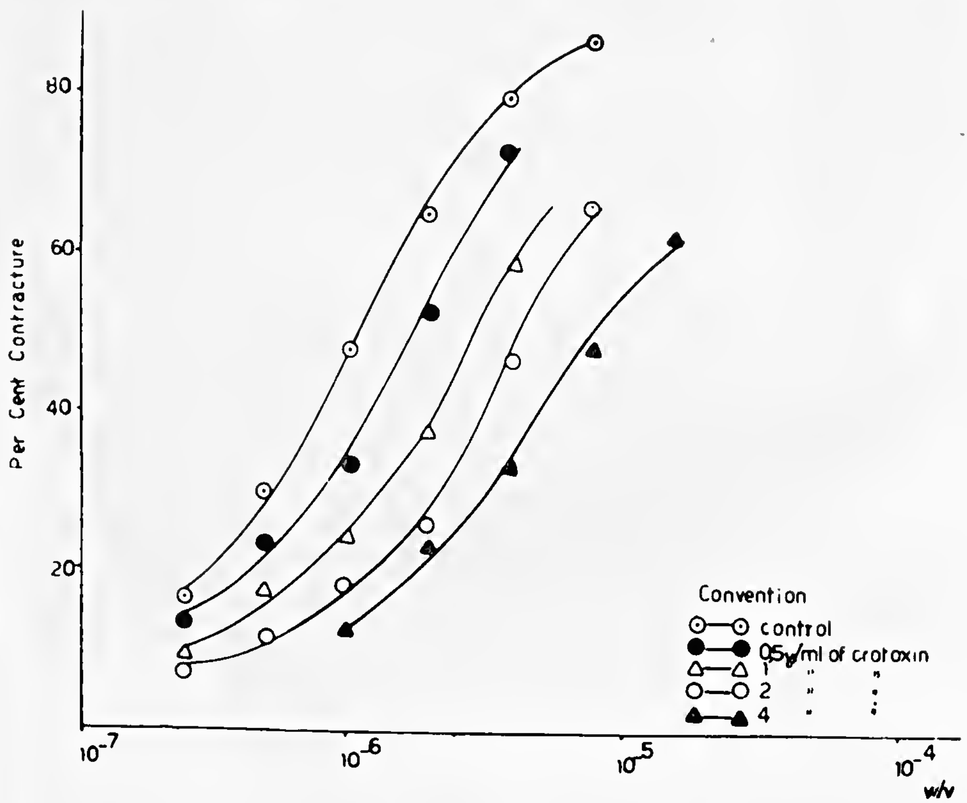

Fig. 5 — Dose-response curves for acetylcholine in the denervated rat hemi-diaphragm preparation in the absence of crotoxin and in the presence of various concentrations (0.5×10^{-6}, 10^{-6}, 2×10^{-6} and 4×10^{-6}) of it. Each dose-response curve was obtained in a diferent denervated diaphragm as the effect evoked by crotoxin was, for pratical purposes, irreversible.

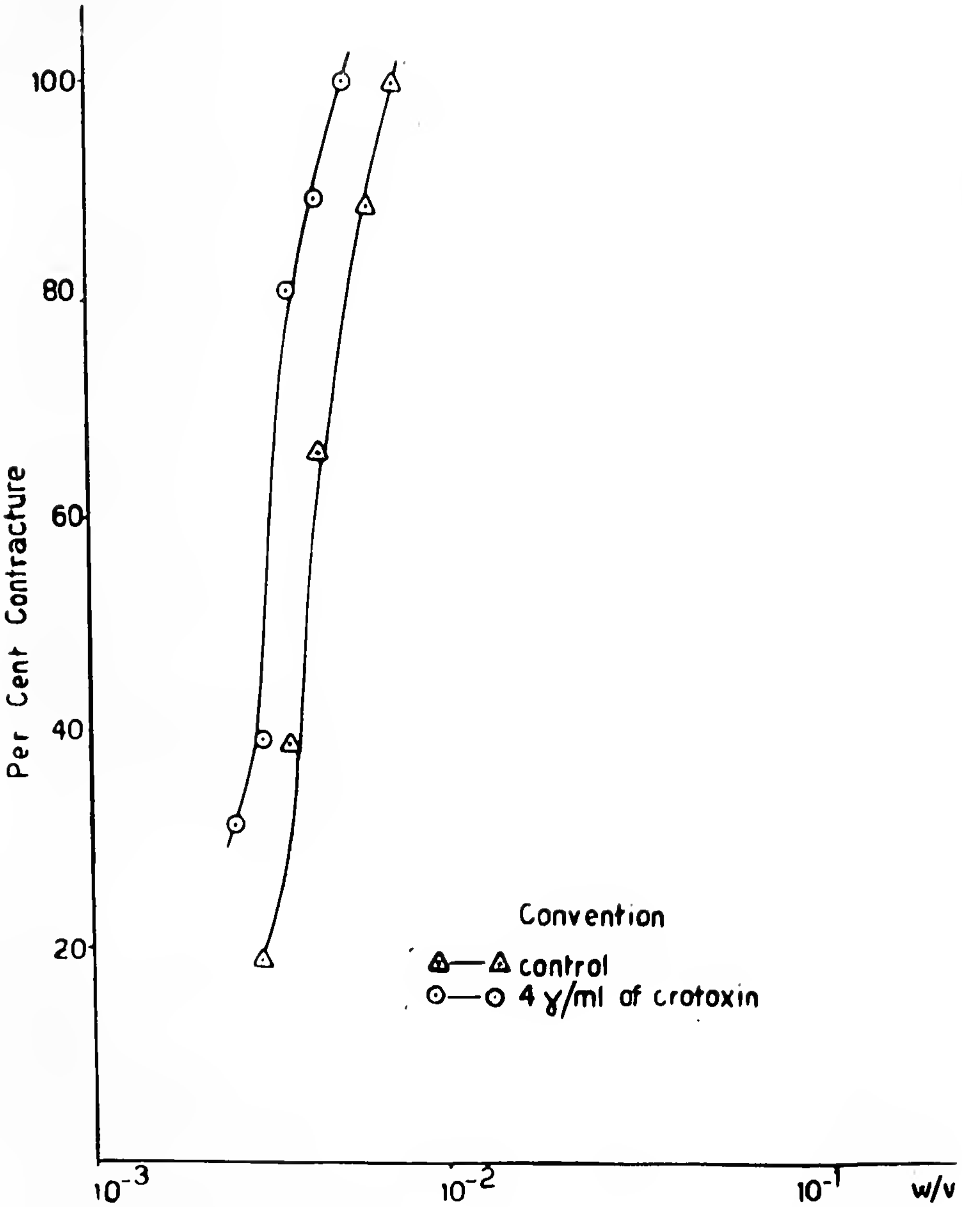

Fig. 6 — Dose-response curves for potassium chloride obtained in the denervated rat hemi-diaphragm in the absence of crotoxin and in the presence of 2×10^{-6} of that substance. Crotoxin potentiated the action of the potassium ions on the denervated muscle.

these animals under crotoxin action were far from resembling those evoked by the depolarizing drugs. On the other hand, crotoxin in large doses produced a flaccid paralysis similar to that evoked by curare while large doses of the depolarizing drugs still give rise to contracture of the skeletal muscles. It seems, therefore, that it can confidently be concluded that crotoxin interrupts neuromuscular transmission by producing a non-depolarization type of blockade.

A non-depolarization block, in its largest acceptation, can have one or more of the following causes: (a) a reduction in the amount of the transmitter liberated by the motor nerve terminals, (b) a decrease of the sensitiveness of the end-plate to the depolarizing action of acetylcholine and (c) a depression of the excitability of the muscle fibre membrane. Botulinum toxin (5), small doses of hemicholinium (6, 7, 8) and the recently studied snake venom components, β- and γ-bungarotoxin (9), for instance, block transmission by the mechanism mentioned in (a) while the competitive neuromuscular blocking drugs and probably α-bungarotoxin (9) by that referred to in (b). A blockade exclusively produced by a depression of the excitability of the muscle fibre membrane is very infrequently observed. However, it seems that an excess of calcium can give rise to a block by such a mechanism,, as the end-plate potencial is not depressed during the blockade elicited by this ion (10). Large doses of procaine and the local anesthetics in general provoke a neuromuscular block which is caused by a combination of the first two or even of all three factors mentioned (11, 12. 13. 14). The same can be said in relation to an excess of magnesium ions (15) and large doses of the antibiotics of the streptomycin group (16, 17, 18, 19), although in these cases the main cause of the blockade certainly is a decrease in the amount of acetylcholine liberated by the nerve terminals. The experiments here reported do not permit to decide if crotoxin diminishes the amount of the transmitter liberated at the end-plate by the nerve impulses. However, it can be inferred from the crotoxin-acetylcholine antagonism revealed in the denervated hemi-diaphragm preparation of the rat that the main cause of the blockade produced by crotoxin is very probably due to a decrease in the sensitiveness of the end-plate to the depolarizing action of acetylcholine. This mechanism of blockade seems also to be demonstrated by the reversion of the succinylcholine effect in cats showing a partial neuromuscular block evoked by crotoxin.

If crotoxin blocks the action of acetylcholine in the denervated muscle and at the end-plates by reacting with the acetylcholine receptors or at some other point of the muscle fibre membrane can not yet be decided. The type of shift produced in the dose-response curve for acetylcholine suggests that both mechanisms of action operate. An action on the acetylcholine receptors is also hinted by the similarity of the order of involvement of the various muscular groups in the animals injected with crotoxin or curare. In any case the fixation of crotoxin on the specific and/or non-specific receptors must not be a loose one as the combination of the majority of biologically active substances with receptors. This seems to be demonstrated by the extreme slowness of the reversibility of the paralysis and hence of the neuromuscular block produced by crotoxin.

Crotoxin exerts little or no effect on transmission at ganglionic synapses as well as at adrenergic neuroeffector junctions. This can be inferred from the results obtained in the experiments here reported concerning the contraction of the nictitating membrane in response to electrical stimulation of the sympathetic cervical chain in cats injected with large doses of crotoxin. As already stated, contractions of the nictitating membrane were always elicited in spite of the fact

that transmission at the myoneural junction was blocked one or more hours before the experiment. It is interesting to note that such selectivity of action on neuromuscular transmission seems to be only displayed by some curarizing drugs or some depolarizing agents. Gallamine and toxiferine, for instance, among the competitive neuromuscular blocking agents only paralyze ganglionic synapses when very large doses of them are administered to cats (20, 21). On the other hand, the agents which interrupts neuromuscular transmission by a prejunctional mechanism such as botulinum toxin, hemicholinium, magnesium or streptomycin are usually quite as active in producing ganglionic blockade as they are in paralysing the myoneural junction.

Summary

1. Similarity of the motor signs evoked by crotoxin and curare suggested the investigation of neuromuscular transmission in animals showing the paralysis elicited by crotoxin. The experiments were carried out on the sciatic-tibialis anterior muscle preparation of dogs to which crotoxin was injected with an antecipation of 24 or 48 hours. A partial or complete block of transmission was revealed in all experiments. Neostigmine, calcium, or bicarbonate were without effect on the blockade. However, the stimulation of the motor nerve with shocks of a high (50 c/s) frequency always caused a transitory post-tetanic improvement on transmission.

2. The neuromuscular blocking action of crotoxin was also studied in cats (sciatic-tibialis anterior muscle preparation). Large doses (0.5 mg/Kg and 1 mg/Kg) were employed. A complete or partial neuromuscular blockade appeared within the first three hours of crotoxin administration. Edrophonium and succinylcholine increased, although only to a small degree, the nearly completely blocked responses of the tibialis anterior muscle. Their effects were transitory.

3. Ganglionic transmission in crotoxin injected cats was investigated by stimulating the cervical sympathetic chain and recording the contractions of the nictitating membrane. The experiments were always done after transmission at the myoneural junction was blocked. The stimulation of the cervical sympathetic chain invariably elicited contractions of the nictitating membrane whose tensions did not decrease during the experiments. It seems, therefore, that no ganglionic blockade was caused by crotoxin.

4. The effect produced by crotoxin on the contractures evoked by acetylcholine or potassium in the isolated and cronically denervated rat hemi-diaphragm was investigated. Crotoxin shifted to the right the dose-response curve for acetylcholine. Only a slight decline of the slopes of the curves obtained in the presence of increasing concentrations of crotoxin ocurred. On the other hand, crotoxin did not antagonize, but potentiated, the action of potassium on the denervated muscle.

5. The following conclusions seem to emerge from the analysis of the results obtained on this investigation: I. Block of neuromuscular transmission is probably the sole cause of the paralysis evoked by crotoxin. II. Crotoxin interrupts neuromuscular transmission by producing a non-depolarization type of blockade. III. A decrease in the sensitiveness of the end-plate to the depolarizing action of acetylcholine is the unique or the main cause of crotoxin neuromuscular blockade. iv. Crotoxin probably reacts with the acetylcholine receptors and also at other sites of the muscle fibre membrane. Its fixation on the receptors can not be a loose one as the combination of the majority of biologically active substances with receptors.

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45. PHARMACOLOGY OF CRYSTALLINE CROTOXIN. III. CARDIOVASCULAR AND RESPIRATORY EFFECTS OF CROTOXIN AND *CROTALUS DURISSUS TERRIFICUS* VENOM.

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The venom of *C. d. terrificus* besides producing skeletal muscle paralysis and renal insufficiency, gives rise to acute disturbances of blood pressure and respiration as it was demonstrated by Arthus (1) and others (2, 3). The main purpose of the present study was to investigate the actions of crotoxin on blood pressure and respiratory function so that the role it represents in the genesis of the disturbances evoked by the venom could be evaluated. Experiments with the venom were also done for adequately evidencing the differences of action and potency between crotoxin and venom.

The mechanism through which acute respiratory disturbances are caused by the venom did not seem to be clear. Houssay (4) concluded from experiments carried out on the perfused and isolated head preparation of dogs that the venom of *C. d. terrificus* exerts direct actions on the central nervous system. He verified that signs of respiratory stimulation and depression appeared in the isolated head following the injection of the venom in the perfuser dog. However, the experiments of Houssay do not eliminate the possibility that the respiratory disturbances could be also reflexly generated through stimulation of chemoreceptors at the carotid and aortic bodies, or of receptors in the lungs. Therefore some experiments were planned to investigate this point. The results suggest that the direct action on the medulla is perhaps less important in causing the acute respiratory effects than the reflex ones.

Material and methods

The venom of *C. d. terrificus* used in this research was extracted as previously reported (5) from snakes captured in Goiás; it belonged to the variety devoid of crotamine. The batches of crotoxin utilized in the experiments were crystalline preparations obtained according to procedures already referred to in the previous paper (5).

This study was supported by a grant from the "Fundação de Amparo à Pesquisa do Estado de São Paulo".

The acute effects evoked by crotoxin and the venom on blood pressure and respiration were compared in dogs anaesthetized with sodium pentobarbital (30 mg/Kg, i.v.). The effect they produced on the haematocrit value was also investigated in most of the experiments. The dogs were prepared for recording arterial blood pressure and respiration as it was described in a previous work (6).

Samples of blood were withdrawn for the determination of the haematocrit values 30 minutes and 1, 2 and 3 hours after the injection of the venom or crotoxin. Twenty-four dogs were employed in the experiments: sixteen animals were injected with crotoxin and eight with the venom. Crotoxin from two batches (crotoxin n.º 5 and n.º 14) was used. No haematocrit value determinations were made in the experiments, in numbers of eight, in which crotoxin n.º 14 was employed. The dose of 0.25 mg/Kg of crotoxin or of the venom was used in all experiments. The arterial blood pressure and respiration were usually recorded for 3 hours after administration of the venom or crotoxin.

The effects produced by crotoxin and the venom were also compared by injecting them in the same dog. Two or three doses of 0.25 mg/Kg, conveniently spaced, of crotoxin were administered in order that the animal become irresponsive to its hypotensive action. Then 0.25 mg/Kg of the venom were injected.

The experiments planned to investigate the mechanisms involved in the genesis of the respiratory effects caused by the venom were carried out in four dogs with both vagi cut, and in ten whose carotid sinus nerves, in addition, were sectioned. Occlusion for 30 or 45 seconds of the common carotid arteries was made before the injection of the venom to test the denervation of the carotid sinus regions. The dogs were anaesthetized with sodium pentobarbital. The dose of 0.25 mg/Kg of the venom was also employed in these experiments.

Results

Table I shows the percentages of the maximums of blood pressure falls observed in the experiments in which crotoxin n.º 5 and crotoxin n.º 14 were used, and their means 32 ± 6.5% and 25 ± 6.44%. The difference between these means is not statistically significant ($P > .05$). Both of them, however, are significantly smaller ($P < .01$) than the mean 58.3 ± 5.6%, obtained from the experiments with the venom *C. d. terrificus* (Table II). The venom was also much more potent in increasing the haematocrit value. Table I and II show the percentages of the increases which were demonstrated 30 minutes after the injection of crotoxin n.º 5 and the venom, as well as their means 20.2 ± 4.4% and 43.5 ± 5.9%. The difference between these means are highly significant ($P << .01$).

The hypotensive effect elicited by crotoxin was always reversible; the arterial blood pressure usually attained its primitive level in less than two hours after its administration. This did not always happen in the experiments with the venom: In two out of eight dogs, the effect on blood pressure was irreversible and the animals died from the hypotension in less than one hour. However, the blood pressure of the other dogs recovered within 2 or 3 hours. The effects evoked by the venom and crotoxin on blood pressure were also qualitatively different from each other (Figs. 1, 2).

TABLE I — MAXIMUMS OF BLOOD PRESSURE DEPRESSIONS AND INCREASES IN THE HEMATOCRIT VALUES CAUSED BY THE INTRAVENOUS ADMINISTRATION OF 0.25 MG/KG OF CROTOXIN

Crotoxin no. 5 *			Crotoxin no. 14 **	
Experiment (no.)	Maximum fall of (%)	Increase in the hematocrit value (%)	Experiment (no.)	Maximum fall of blood pressure (%)
1	40.9	7.5	1	29
2	30.6	17.0	2	15
3	56.2	40.0	3	35
4	37.7	17.0	4	0
5	19.3	12.0	5	10
6	14.2	17.8	6	50
7	55.5	39.4	7	49
8	6.5	10.6	8	17
	Mean ± S.E.	Mean ± S.E.		Mean ± S.E.
	32.6 ± 6.5%	20.2 ± 4.4%		25.6 ± 6.44%

* LD_{50} for mice (i.v.) 82 (66.9 to 101.4) mcg/Kg.
** LD_{50} for mice (i.v.) 80.3 (60.8 to 108.9) mcg/Kg.

TABLE II — MAXIMUMS OF BLOOD PRESSURE DEPRESSIONS AND INCREASES IN THE HEMATOCRIT VALUES CAUSED BY THE INTRAVENOUS ADMINISTRATION OF 0.25 MG/KG OF THE VENOM OF *C. D. TERRIFICUS*

Experiment (no.)	Maximum fall of blood pressure (%)	Increase in the hematocrit value (%)
1	37.7	44.7
2	71.7	50.0
3	70.0	44.2
4	73.3	21.5
5	50.7	35.1
6	60.2	56.4
7	69.7	71.7
8	33.3	24.3
	Mean ± S.E.	Mean ± S.E.
	58.3 ± 5.6%	43.5 ± 5.9%

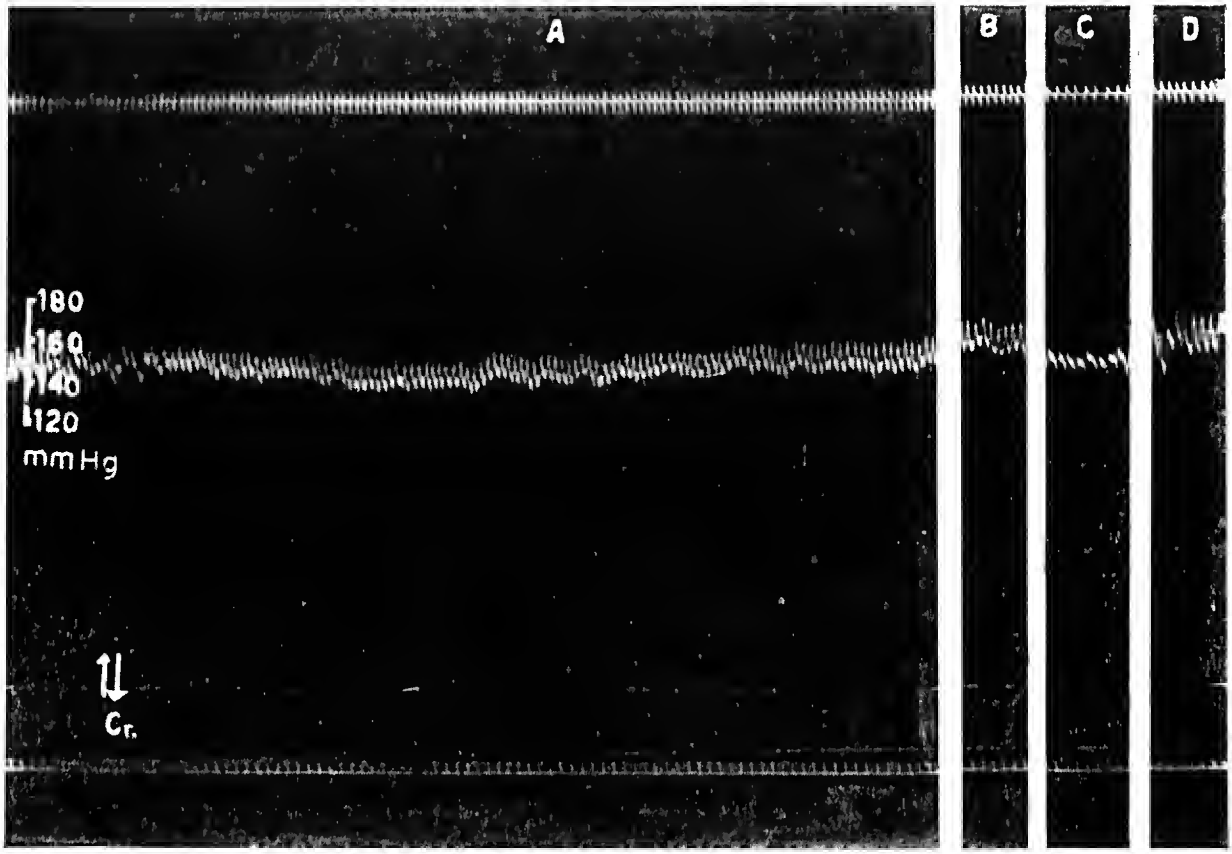

Fig. 1 — Dog, 10.5 Kg, sodium pentobarbital. Records of respiration and blood pressure. At Cr., 0.25 mg/Kg of crotoxin were injected. At B, C and D, records taken 30, 60 and 90 minutes after A. Time 6/6 seconds.

Crotoxin produced a rather gradual blood pressure fall which began to appear from 2 to 3 minutes after its administration. After the venom, on the other hand, an abrupt and transient fall of blood pressure appeared within the first 30 seconds and was followed by a pressor effect of one or two minutes duration. Thereafter a less abrupt and prolonged fall occurred in all experiments. It was usually more intense than the first fall.

Respiration did not seem to be acutely modified by crotoxin (Fig. 1). The venom, on the other hand, caused an intense and transient increase in the frequency and amplitude of the respiratory movements a few seconds after its administration. This was followed by apnoea of brief duration and then by tachypnoea which persisted for more than one hour (Fig. 2).

Haemolysis was demonstrated in all experiments in the blood extracted two hours after the injection of crotoxin or venom.

The striking differences in the effects elicited by crotoxin and venom were also revealed by injecting them in the same dog. The venom caused the usual disturbances on blood pressure (and also on respiration) after the dog was made irresponsive (tachyphylaxis) to the hypotensive action of crotoxin. Fig. 3 shows the records of an experiment in which the blood pressure was not altered by crotoxin. Nevertheless, the venom produced, as usually, the characteristic effects on blood pressure and respiration.

The apnoea usually elicited by the venom did not occur after its administration in the dogs whose vagi had been cut. In its place, a brief phase of decreased respiratory excursions was observed (Fig. 4). The other acute respiratory disturbances evoked by the venom were definitely attenuated in all experiments. In eight out of the ten dogs whose vagi and carotid sinus nerves had been sectioned, the venom gave rise to only a slight increase in the respiratory frequency and to a very brief phase in which a small diminution in the respiratory amplitude occurred. In two of these animals the respiratory effects were almost suppressed (Fig. 5). On the other hand, in two other experiments the respiratory disturbances were nearly as intense as those observed in dogs with the vagi and carotid sinus nerves intact. However, the apnoea did not occur.

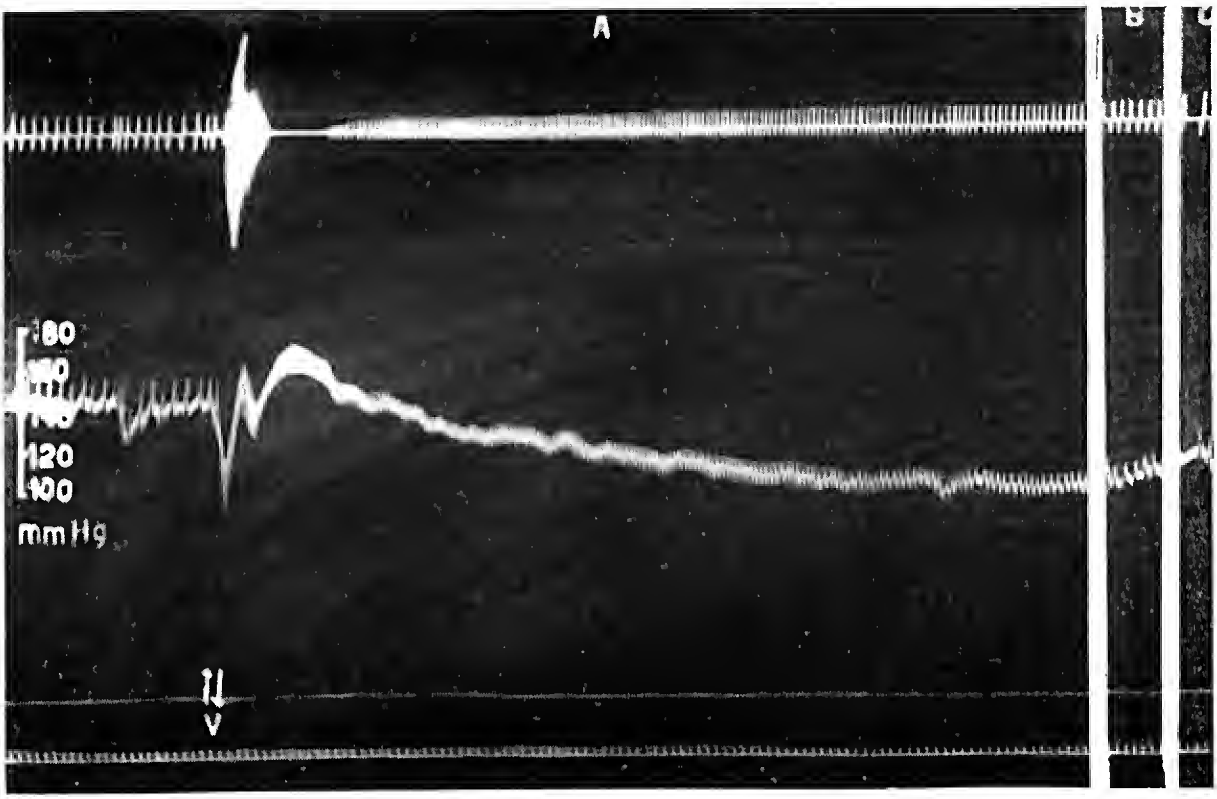

Fig. 2 — Dog, 7 Kg, sodium pentobarbital. Records of respiration and blood pressure. At V., 0.25 mg/Kg of *C. d. terrificus* venom were injected. At B, C and D, records taken 30, 60 and 150 minutes after A. Times 6/6 seconds.

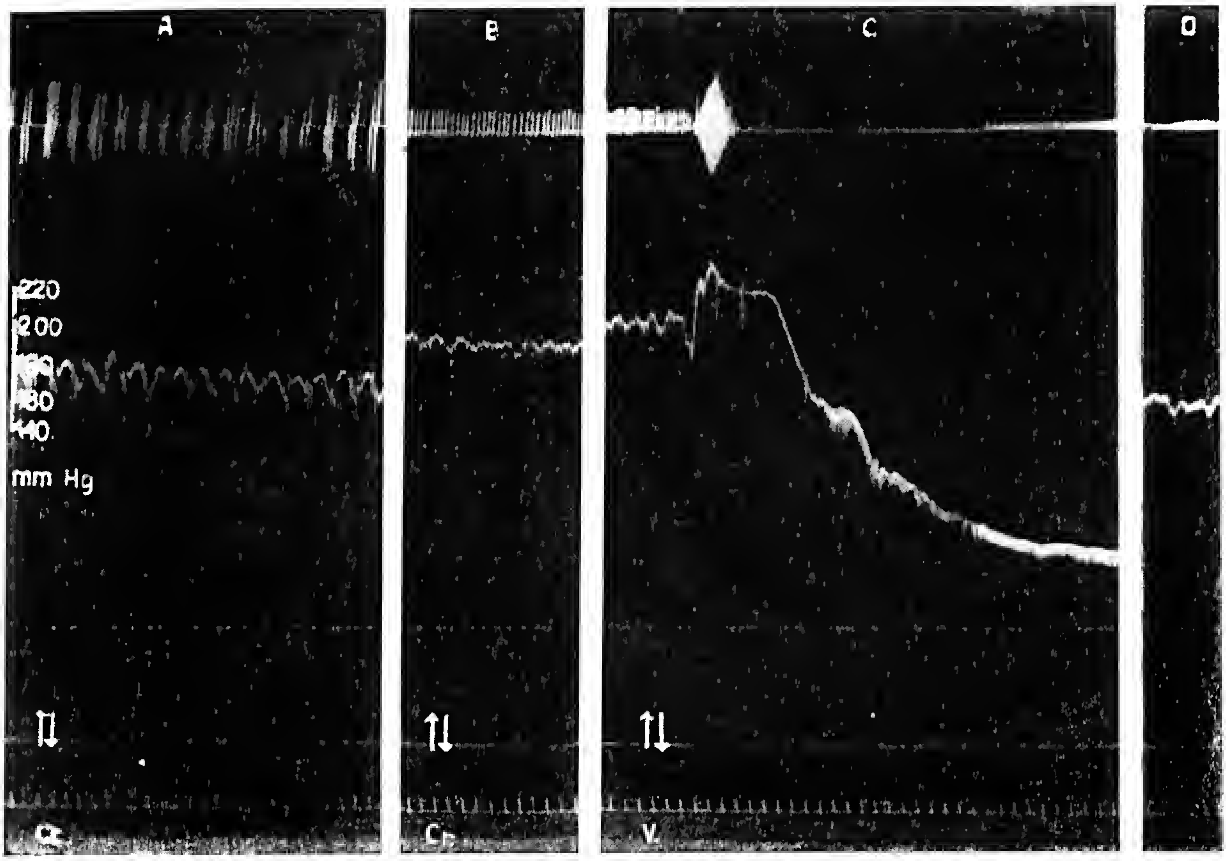

Fig. 3 — Dog, 10 Kg, sodium pentobarbital. Records of respiration and blood pressure. At Cr. (panel A and B), 0.25 mg/Kg of crotoxin were injected; at V. (panel C), 0.25 mg/Kg of the venom of *C. d. terrificus* were administered. At B, C and D, records taken 1, 2 and 3 hours after A. Time 6/6 seconds.

Discussion

The results of the present investigation show that the remarkable acute disturbances provoked by the venom of the South American rattlesnake on circulation and respiration are not due to crotoxin. Neither can they be attributed to crotamine because the venom employed in the experiments did not contain that toxin. Therefore, it can be inferred that, besides these, there are other components of the venom of *C. d. terrificus* whose pharmacological actions must play an important role in clinical envenomation, especially in the genesis of shock, a condition frequently exhibited by the patients bitten by that snake (7). The separation as well as the pharmacological and immunological study of such components is highly desirable.

It is surprising that, being endowed with phospholipase A activity, crotoxin exhibits so small hypotensive and shocking potencies. Lysolecithin, in effect, besides being hemolytic, produces in dogs and cats an abrupt fall of blood pressure and releases histamine (8). Feldberg, Holden and Kellaway (8) attributed

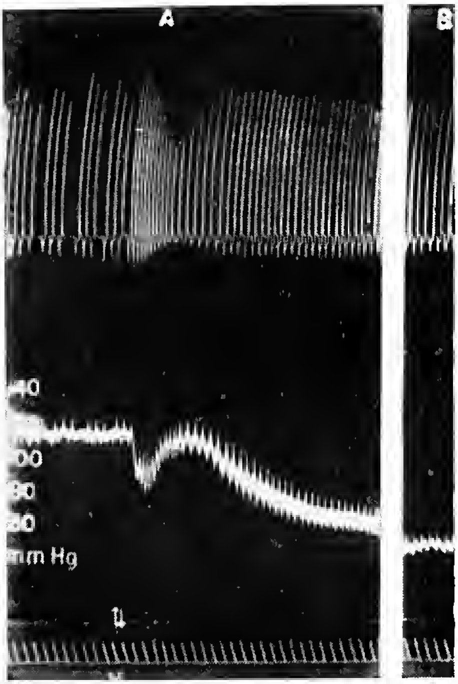

Fig. 4 — Dog, 6.0 Kg, sodium pentobarbital, both vagi sectioned. Records of respiration and blood pressure. At arrows, 0.25 mg/Kg of the venom of *C. d. terrificus* were injected. At B, record taken 7 minutes after A. Time 10/10 seconds.

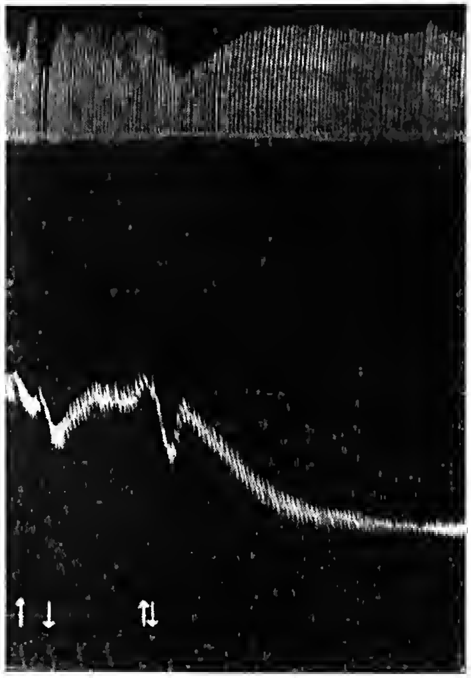

Fig. 5 — Dog, 6.5 Kg, sodium pentobarbital, both vagi and carotid sinus nerves sectioned. Records of respiration and blood pressure. First arrows, compression of the common carotid arteries was made, second arrows, 0.25 mg/Kg of the venom of *C. d. terrificus* were injected. Time 10/10 seconds.

much of the cardiovascular effects produced by the Indian cobra (*Naja naja*) venom to lysolecithin formation, an interpretation which can perhaps be questioned in view of the present results.

The triple effect, here reported, produced by the venom of *C. d. terrificus* on blood pressure, that is, the transient and abrupt fall of blood pressure which is followed by an hypertensive period of short duration and a secondary depression, was first described by Arthus in rabbits (1). He reported that he did not observe such a sequence of effects after administration of the other venoms he had studied. Vellard and Huidobro, on the other hand, showed that the venom of *C. d. terrificus* from some regions produces only depression of blood pressure, a finding that was confirmed by one of us (3) with the venom from rattlesnakes of Santiago del Estero, Argentina. The triple effect, as previously shown (3), is not supressed in dogs (i) injected with atropine, (ii) with their central nervous system destroyed by the procedure of Galvão and Pereira, (iii) with their autonomic ganglia blocked by hexamethonium and finally (iv) with their α adrenergic receptors blocked by dibenamine or chlorpromazine.

The brief period of apnoea can not be imputed to the respiratory stimulation caused by the venom as it was also observed in experiments in which only a small stimulation of the dog respiration was elicited. Moreover, in the experiments in which both vagi had been cut, the apnoea did not occur even in those

dogs whose respiration was intensely stimulated (two experiments in animals with the vagi and carotid sinus nerves cut). It seems, therefore, that this apnoea originates from the stimulation, in the lungs, of vagus nerve fibres which conduct inhibitory impulses to the respiratory centers. The brief period of apnoea evoked by nicotine also arises, according to Aviado (9), from stimulation of receptors in the lungs.

The respiratory stimulation caused by the venom was, in most cases, greatly reduced in those dogs whose vagi and carotid sinus nerves had been sectioned. This suggests that is must be, in part at least, reflexly generated, probably by an action of the venom on the chemoreceptors of the carotid and aortic bodies.

Summary

1. A comparative study of the acute effects produced by crotoxin and the venom of *C. d. terrificus* on blood pressure, haematocrit value and respiration was carried out in sodium pentobarbital anaesthetized dogs. The venom used in the investigation belonged to the variety devoid of crotamine.

2. Crotoxin was much less active than the venom in producing hypotension and in increasing the haematocrit value. The effects elicited by them on blood pressure were also qualitatively different from each other.

3. Respiration was not acutely modified by crotoxin. The venom, on the other hand, caused an intense and transient increase in the frequency and amplitude of the respiratory movements within a few seconds after its administration, a brief period of apnoea and one of tachypnoea which usually was very long lasting.

4. The results of the present investigation show, therefore, that the acute disturbances elicited by the venom on circulation and respiration can not be due to crotoxin or to crotamine. There are other venom components whose pharmacological actions must play an important role in *C. d. terrificus* envenomation, especially in the genesis of shock.

5. Some experiments were also made to clear up the mechanisms involved in the genesis of the respiratory disturbances that follow the administration of the venom. It was inferred from the results of these experiments that the brief period of apnoea originates from stimulation of receptors in the lungs while the respiratory stimulation is due to an activation of the chemoreceptors in the aortic and carotid sinus bodies, and to a direct action on the respiratory centers.

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46. PHARMACOLOGY OF CRYSTALLINE CROTOXIN. IV. NEPHROTOXICITY

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Renal lesions identical to those originally found in crush syndrome (haemoglobinuric nephrosis, lower nephron nephrosis or tubulorhexis) have been described in autopsy and biopsy tissue (1, 2, 5) from human cases of snakebite caused by *Crotalus durissus terrificus* (South American rattlesnake). Similar lesions, according to Amorim and coworkers (3) can be experimentally produced by injecting dogs with the crude venom of this snake. Identification of the component or components of the venom responsible for such histopathologic picture is highly desirable. A study was, therefore, undertaken of the renal lesions produced in dogs by crotoxin, one of the main toxins of the venom. Besides motor paralysis, this venom component elicits albuminuria, haemoglobinuria and olyguria in dogs as it was shown in a previous paper (7).

Material and Methods

The histopathological study was made on the kidneys of twenty dogs intravenously injected with doses of 0.102, 0.128, 0.160, 0.200 and 0.250 mg/Kg of crotoxin. The kidneys were removed one to nine days after crotoxin administration from animals under sodium pentobarbital anaesthesia or from dogs recently died of crotoxin intoxication.

Pieces of the kidneys were fixed in Bouin's fluid, or in 10% formalin in phosphate buffer (pH 7.0), included in paraffin, cut and stained by haemotoxylin and eosine or Masson's trichrome method. PAS, and benzidine reaction for haemoglobin detection (5), were also made.

Results

Both glomeruli and renal tubules showed pathological alterations. There was some correlation between intensity of lesions and the dose of crotoxin injected, the lesions being more marked in the kidneys of those animals which received higher doses. There were, however, some variations not correlated to the doses.

This study was supported by a grant from the "Fundação de Amparo à Pesquisa do Estado de São Paulo".

The renal lesions showed focal distribution: injuried areas were always found to be separated by apparently normal ones.

It was possible to recognize some differences between early lesions, which occurred in the first four days following crotoxin injection, and late lesions, observed after the fourth day. Such differences concern the component of the nephron most attained by the lesions. In early lesions, the renal tubules were less attained than the glomeruli; in late ones, tubular lesions predominated.

Early lesions

Glomerulus. The lesions most constantly observed were capillary congestion, thickness of the basement membrane, deposit of PAS positive material between the capillary loops (Fig. 1) and nuclear pycnosis of some glomerular cells.

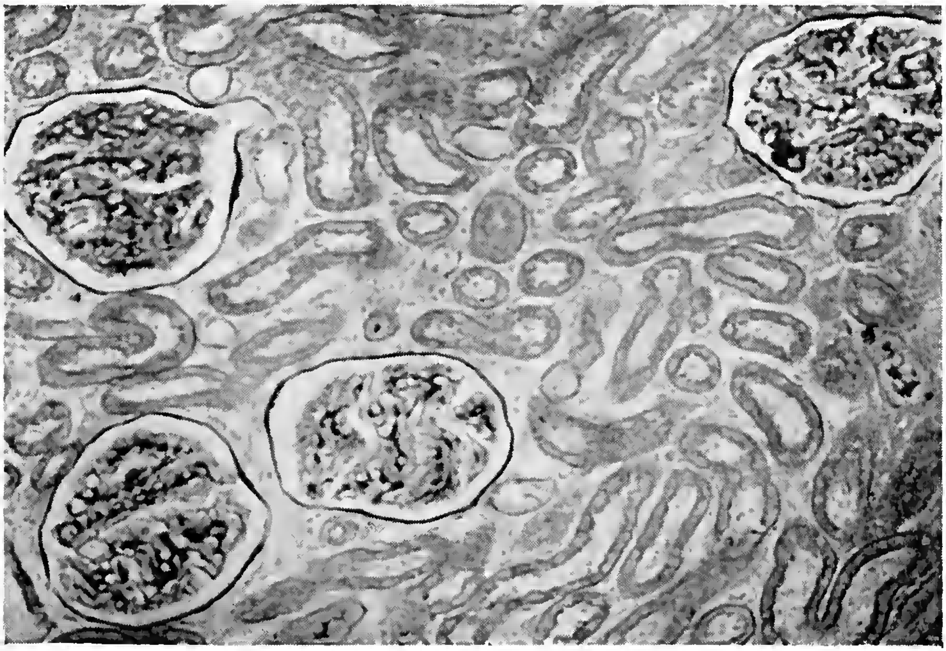


Fig. 1 — Section of kidney of dog injected with crotoxin. PAS reaction. Early lesions. Cross section of glomeruli shows thickness of the basement membrane and some amorphous PAS positive material among the glomerular loops.

Proximal tubule. Degenerative lesions were present in some epithelial cells of the proximal convolutions. The brush border of the damaged cells desappeared at the same time that an alteration of the normal distribution as well as a numerical reduction of the mitochondria occurred (Fig. 2). The cytoplasm of the damaged cells appeared vacuolated and the nucleus was sometimes pycnotic.

It must be emphasized that the degenerative lesions reached only some tubular cells, in very restricted areas. These areas were situated chiefly in the internal zone of the renal cortex, corresponding to the terminal portion of the proximal convoluted tubule.

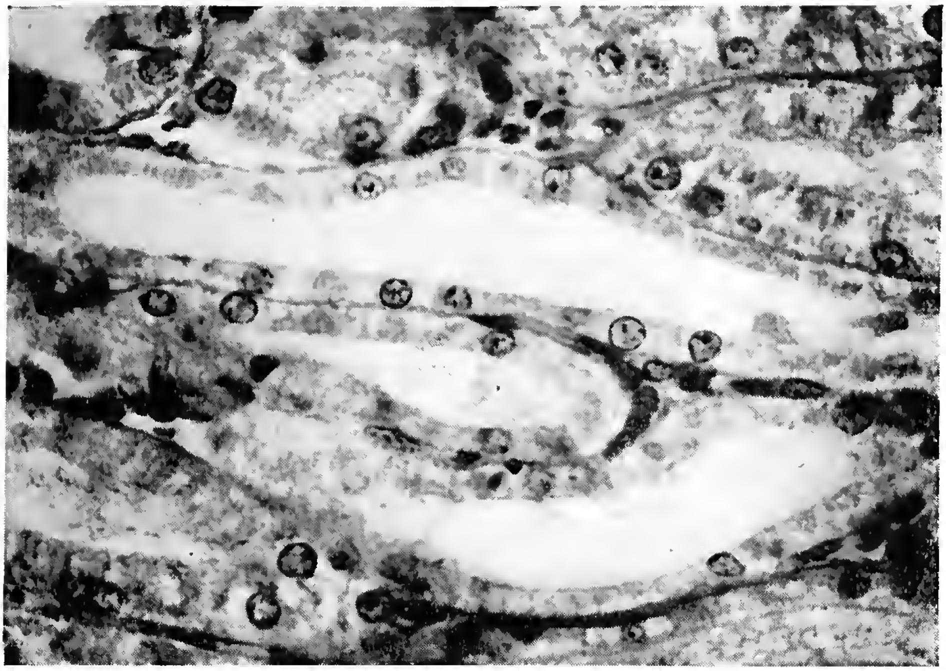


Fig. 2 — Section of kidney of dog injected with crotoxin. Masson's trichrome stain. Late lesions. The degenerative lesions predominate in proximal tubules whose cells lost their brush border and present little amount of irregularly distributed mitochondria.

Inside the tubules, it was frequently found PAS positive and haematic casts.

Distal tubule. Degenerative lesions similar to those described for the proximal tubular cells were found in distal tubules. However, the injuried areas were smaller and the damaged cells less numerous than those in the proximal convolutions.

Collecting ducts. The collecting duct cells were apparently normal. In the collecting duct lumen, some PAS positive haematic casts could be seen.

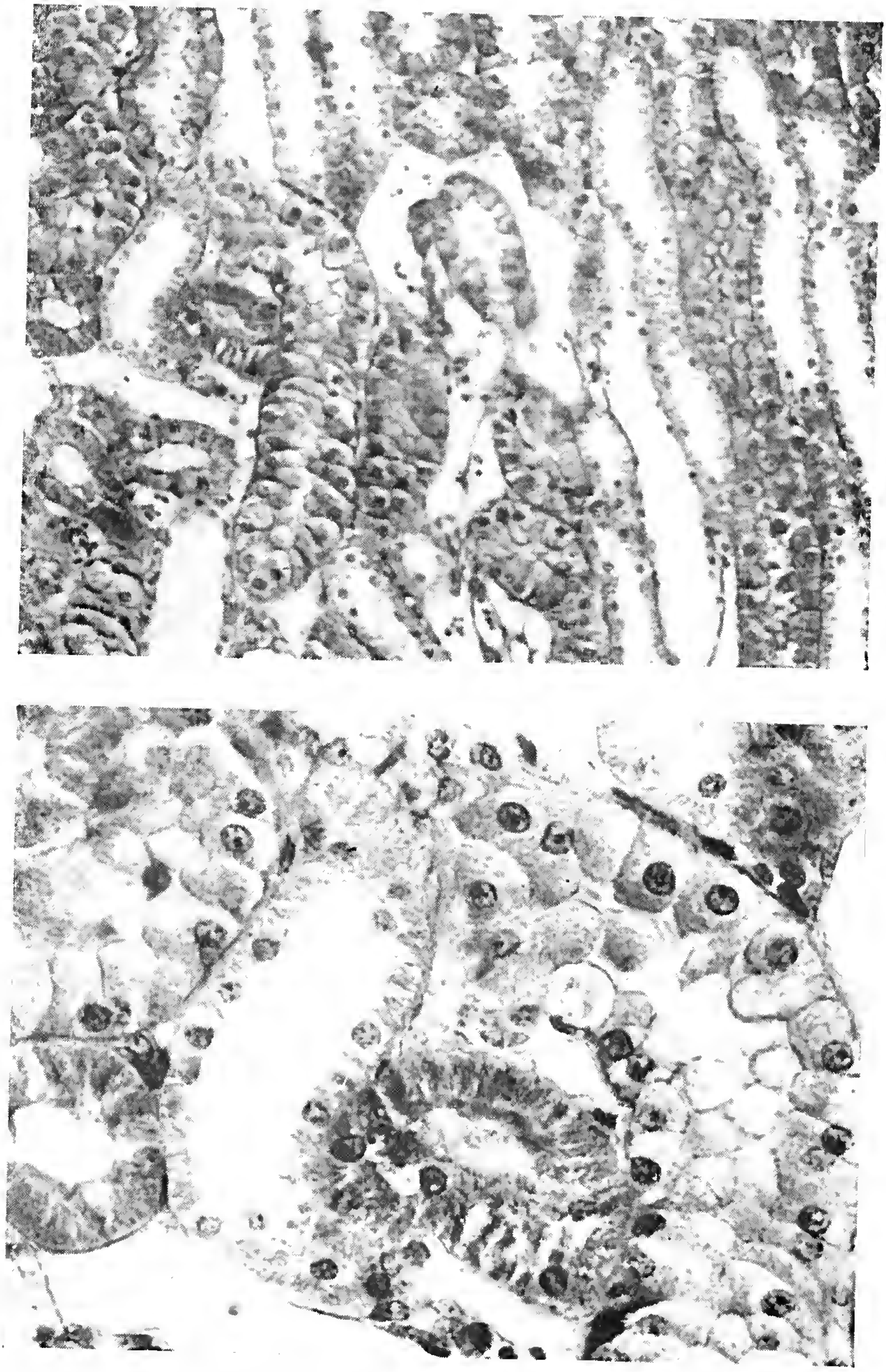
Interstitial tissue. Some areas of oedema near the injuried tubules were observed.

LATE LESIONS

Glomerulus. The glomeruli showed in most cases a normal aspect. However, thickness of the basement membrane could be observed in a few glomeruli.

Proximal tubule. The proximal tubules were intensely injuried. Although focal in character, the lesions had a tendency to become diffuse through confluence of neighboring injured areas.

The lesions predominated in terminal segments of the proximal tubules. However, cells in the initial ones were also sometimes damaged.



Figs. 3 and 4 — Section of kidney of dog injected with crotoxin. Masson's trichrome stain. Late lesions. The proximal tubules show strongly swollen cells without brush border. The distal tubule cells as well as those of some proximal convolutions present only minor lesions. In this instance,, the cells keep their brush border and mitochondria pattern.

The fellowing alterations could be seen in the epithelial cells of the damaged tubules: disappearence of their brush border, a great decrease in the amount as well as an irregular pattern of the mitochondria, swelling and microvacuolar degeneration of the cytoplasm. When intense this degenerative process gave a foamy aspect to the cells (Figs. 3, 4, 5 and 6). The intensely vacuolated cells showed nuclear pycnosis and necrosis. Some PAS positive granules could be observed in the cytoplasm of the vacuolized cells.

PAS positive casts were seen in the lumen of the proximal tubules.

Distal tubule. The lesions were qualitatively identical to those seen in the proximal tubules. However. they were much less intense, cellular necrosis being rarely seen. Disorganization of the mitochondria pattern and slight microvacuolization of the cytoplasm of the epithelial cells (Figs. 4 and 5) were the predominant alterations observed.

The initial portion of the distal tubules appeared more intensely damaged than the distal one.

Collecting ducts. Collecting ducts did not show lesions. In their lumen some PAS positive casts could be seen.

Interstitial tissue. Around the injured tubules there was some chronic inflamatory infiltration and sometimes tissue neoformation.

Fig. 5 — Section of kidney of dog injected with crotoxin. Masson's trichrome stain. Late lesions. Cells of proximal convolutions show intense microvacuolar degeneration. In distal tubules the mitochondria pattern is altered and discret microvacuolar degeneration of their cells can be seen.

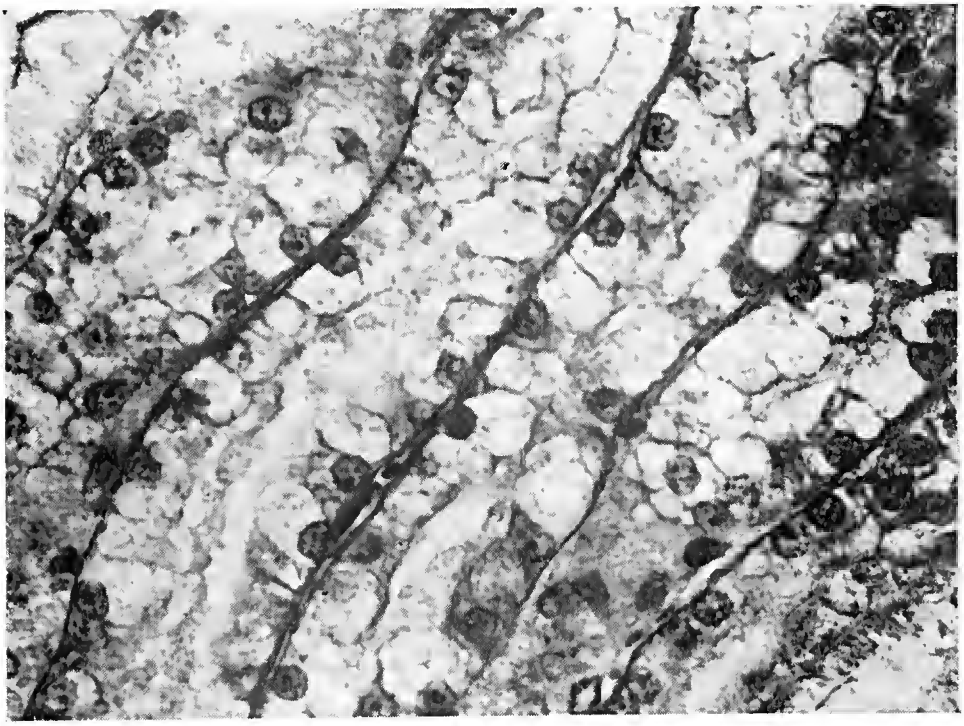


Fig. 6 — Section of kidney of dog injected with crotoxin. Haematoxylin and eosine. Late lesions. Intense microvacuolar degeneration and cellular necrosis of proximal convoluted tubules can be seen.

Discussion

The histopathological picture shown by the kidneys of dogs injected with crotoxin was not exactly the same as that which Amorim and Mello (1, 2) described in human cases of *C. d. terrificus* snakebite. These authors stated, in effect, that the ascending limb of the Henle's loop and the distal convoluted tubule were the predominant sites of the degenerative and necrobiotic lesions. In the kidneys from the dogs intoxicated with crotoxin, on the other hand, the most attained segment of the renal tubule was the proximal one. In both cases, however, the lesions presented a focal distribution. Amorim and coworkers (3) also found the ascending limb of the Henle's loop and the distal convolution to be the prevailing situation of the renal lesions of dogs injected with the South American rattlesnake venom: "The histological lesions of the tubules are principally characterized by degenerative and more serious necrobiotic lesions in the so-called intermediated segment of the nephron, i.e. the ascending branch of Henle's loop and of the distal or secondary convoluted tubule predominanting in the boundary zone of the kidney" (3). However, Rodrigues Lima (6) found in biopsy tissue from human cases of *C. d. terrificus* envenomation that degenerative lesions predominated in the proximal convolutions while necrotic ones were more frequent in the distal tubules: "Nos nossos casos, o néfron foi lesado como um todo, embora as lesões degenerativas tenham preferência por túbulos proximais, enquanto que a necrose e a regeneração ocorreram com mais freqüên-

cia no segmento distal" (6). The degenerative lesions of the tubular epithelium of the proximal convolutions were, as described by Rodrigues Lima (6), of the same type of those we found in the kidneys of dogs injected with crotoxin: "Estas lesões (the degenerative lesions) têm aspectos de microvacúolos que rechaçam o núcleo para a periferia, tomando total ou parcialmente o citoplasma celular ou apresentam fina granulação citoplasmática com borramento dos limites celulares. Êstes aspectos foram vistos com maior freqüência nos túbulos contornados proximais" (6).

Three main factors are probably at work in generating the lesions found in envenomation caused by *C. d. terrificus* snakebite, and in crotoxin intoxication. They are (1) nephrotoxic substances, (2) intravascular haemolysis and (3) shock. The haemolytic activities of crotoxin and of the venom are nearly the same. They are due to their phospholipase A activities, i. e. to lysolecithin formation. Thus the factor mentioned in (2) can not explain the differences observed in the renal histopathologic picture of the two conditions.

Lysolecithin besides being haemolytic is toxic to renal epithelial cells. It is formed by crotoxin, which is composed of crotactin (a polypeptide toxin) bound to phospholipase A (4), and by the venom through its content of crotoxin and, perhaps, free phospholipase A. The local formation of lysolecithin at the surface of the tubular epithelium could be one of the causes of tubular lesions, mainly those of proximal convolutions. This local formation of lysolecithin seems to be very probable since crotoxin attains very high concentrations in the kidneys [a fact which has been revealed in experiments with ^{131}I-labelled crotoxin (unpublished data)]. Thus the nephrotoxic factor would play a more important role in crotoxin intoxication than in the envenomation caused by *C. d. terrificus* venom. This hypothesis explains why the lesions of proximal convolutions are prevalent in crotoxin damaged kidneys.

Shock is the main factor responsible for renal ischaemia, which, in turn, is the cause of the lesions typical of tubulorhexis. Crotoxin was found to be much less potent than the venom in eliciting hypotension and haemoconcentration (8), i. e., the venom is much more active in producing shock. This fact seems to explain why the renal histopathologic picture of the envenomation is more close than that of crotoxin, to the histopathologic picture of the acute renal failure known as lower nephron nephrosis or tubulorhexis.

Summary

An investigation of the renal lesions produced by crotoxin was carried out on the kidneys of twenty dogs intravenously injected with this substance. The kidneys were removed for histological section one to nine days after crotoxin administration from animals under pentobarbital anaesthesia or from dogs recently died of crotoxin intoxication.

Early lesions which occurred in kidneys of dogs within the first four days following crotoxin injection, differed somewhat from late lesions observed after the fourth day.In early lesions the renal tubules were less attained than the glomeruli which showed capillary congestion, thickness of the basement membrane, deposit of PAS positive material between the capillary loops and nuclear pycnosis of some glomerular cells. The degenerative lesions of the proximal convolutions reached only some epithelial cells in very restricted areas. Damaged cells in distal convolutions were still less numerous. In late lesions, tubular damage

predominated, the segment of the tubules most attained being the proximal one. They showed intense microvacuolar degeneration as well as nuclear pycnosis and necrosis of many epithelial cells.

Early or late lesions showed a focal distribution. However, in late lesions those of the proximal convolutions had a tendency to became diffuse through confluence of neighboring injuried areas.

The renal histopathological picture of crotoxin intoxication was compared with that presented by kidneys of human beings bitten by the South American rattlesnake (*Crotalus durissus terrificus*) and of dogs injected with its venom. An hypothesis was formulated to explain the differences observed.

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47. PHARMACOLOGICAL PROPERTIES OF CARDIOTOXIN ISOLATED FROM THE VENOM OF *NAJA NAJA ATRA* *

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Lyophylized venom of *Naja naja atra* was fractionated on columns of CM-Sephadex (G-50) into 13 fractions by gradient elution with ammonium acetate buffer at pH 5.7. Among them five fractions (V-IX) were found to be neurotoxic and three (X, XII, XIII) were cardiotoxic. Intraperitoneal LD_{50} in mice was 0.074 μg/g for Fr. VIII — the major neurotoxic component (NT) and 1.48 μg/g for Fr. XIII — the major cardiotoxic one (CT). CT caused contracture, as well as reduction of resting membrane potentials, of the frog's sartorius, chick's biventer cervicis, and rat's diaphragm. In the absence of calcium, the contracture was markedly reduced, although the depolarizing effect remained unchanged. Neither contracture nor depolarization was caused by NT. The terminal nerve spikes of the frog sartorius were abolished by CT but unaffected by NT. CT caused systolic arrest of isolated frog hearts and rat's atria by reducing the membrane potentials, whereas NT was almost without effect up to 10^{-4} g/ml. CT caused a slow contraction of the guinea pig ileum, which was partially antagonized by either atropine or procaine but not by hexamethonium or antihistaminics. In the presence of CT (10^{-6} — 10^{-5} g/ml), the responses to nicotine and 5-hydroxytryptamine were greatly inhibited, usually preceded by an initial and transient facilitation. The responses to histamine and acetylcholine were not, or only slightly, reduced by CT. The vessels of the rabbit ear were constricted by CT. In cats, CT caused a fall in systolic pressure more than diastolic pressure, accompanied by various ECG changes, such as P-R interval prolongation, inverted T waves, S-T segment depression, ventricular premature beats, A-V interference, complete A-V block, idioventricular rhythm etc. It is concluded that cardiotoxin isolated from cobra venom acts on various excitable cells, predominantly, if not entirely, by reducing the membrane potentials.

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* Aided by the U. S. Army Med. Res. Develop. Command Research. Grant DA — MD — 49 — 193 — 64 — G 108 and by the National Council on Science Development, Republic of China.

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48. THE ACTION OF STAPHYLOCOCCAL ALPHA TOXIN AND THE VENOM FROM *BITIS GABONICA* ON RAT STOMACH STRIP

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Institute of Pharmacology Czechoslovak Academy of Sciences, Prague

We are most grateful to be allowed to speak before this most distinguished community concerned with the research of animal venoms. We feel a little embarrassed as poor relatives concerned only with bacterial toxins. In general two types of venoms are treated by entirely different groups with no close relation between them. We hope that our modest contribution might arouse some interest to strenghten the cooperation between workers in the two subjects.

In our laboratory we are interested in the pharmacology of Staphylococcal alpha toxin. From your point of view older findings of the group of North and Doery are very interesting. They demonstrated a protection against Staphylococcal alpha toxin by pretreating the animals with tiger snake venom (1). They suggested that the snake venom and Staphylococcal alpha toxin occupied the same type of receptors. Their experiments point to the probability that the actual protective effect is produced by phospholipase A. Long chain unsaturated fatty acids are liberated and lysophosphatide is formed. Ganglioside has the same protective action against Staphylococcal alpha toxin. The group suggested that phospholipase A plays a role in animal defence against bacterial exotoxins. This points to a possible common mechanisms between animal and bacterial toxins and therefore we looked for suitable models where some similarities and differences could be demonstrated. Originally we planned to use for this demonstration isolated mast cells, however, according to the results we have up to now, the correlations on this model were not so easy to demonstrate. We have therefore taken the liberty to discuss our results on another model, which is not in the original title of our communication.

Material and Methods

Rats of both sexes were fasted over night, killed by decapitation and isolated rat stomach strip preparations using Vane's method (2) were set up. The stomach strips were suspended in 5 ml bath with Krebs solution at 37°C, and bubbled with air.

Two batches of Staphylococcal alpha toxin were used one crude product and one of 70% purity (gift from Dr. Bernheimer). *Clostridium perfringens* toxin was a product of GLAXO laboratories. Venom from *Bitis gabonica* was obtained through generosity of Dr. Kornalík.

Technical assistance: B. Rybová, and O. Fialová.

Results

Crude or purified Staphylococcal alpha toxin elicited on isolated rat stomach strip a contraction which has a two phase character. After the toxin application a rapid contraction developed which started to decline about 60 sec after and then a new slow, long lasting contraction developed. With repeated toxin application the rapid phase of the contraction did not appear any more and the slow reaction was of lower intensity than the first time. Fig. 1 demonstrates the

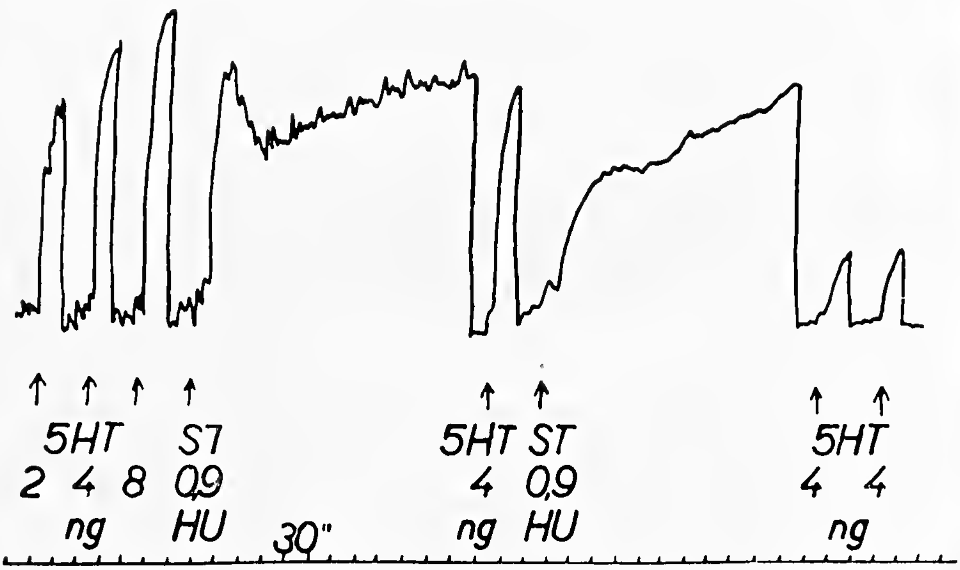

Fig. 1 — Effect of Staphylococcal (ST) alpha toxin on isolated rat stomach preparation. Doses are in nanograms (ng) or hemolytical units (H.U.) per ml of bath.

results. When the repetition of toxin application was continued, the sensitivity of the organ to toxin or 5HT declined and finally vanished. The organ became also unresponsive to 5HT administration. In the next series of experiments the contraction produced by Staphylococcal alpha toxin was compared with the effect of *Bitis gabonica* venom. This venom elicited a rapid contraction which gradually declined even without washing. With repeated doses the organ became

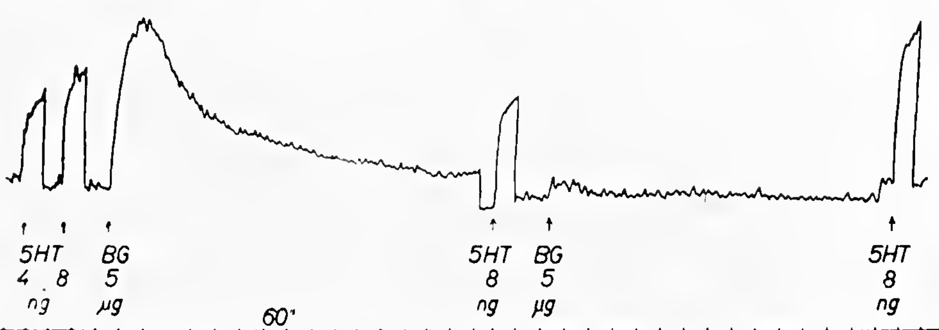

Fig. 2 — Effect of snake venom from *Bitis gabonica* (BG) on isolated rat stomach preparation.

insensitive to further administration of snake venom. On the other hand both serotonin and Staphylococcal alpha toxin elicited a contraction when administered after the snake venom but the character of contraction differs. The first rapid phase is not so evident (Figs. 2 and 3). As may be see from Fig. 4, Staphylococcal alpha toxin administered to the bath rendered the organ almost insensitive to *Bitis gabonica* venom administration.

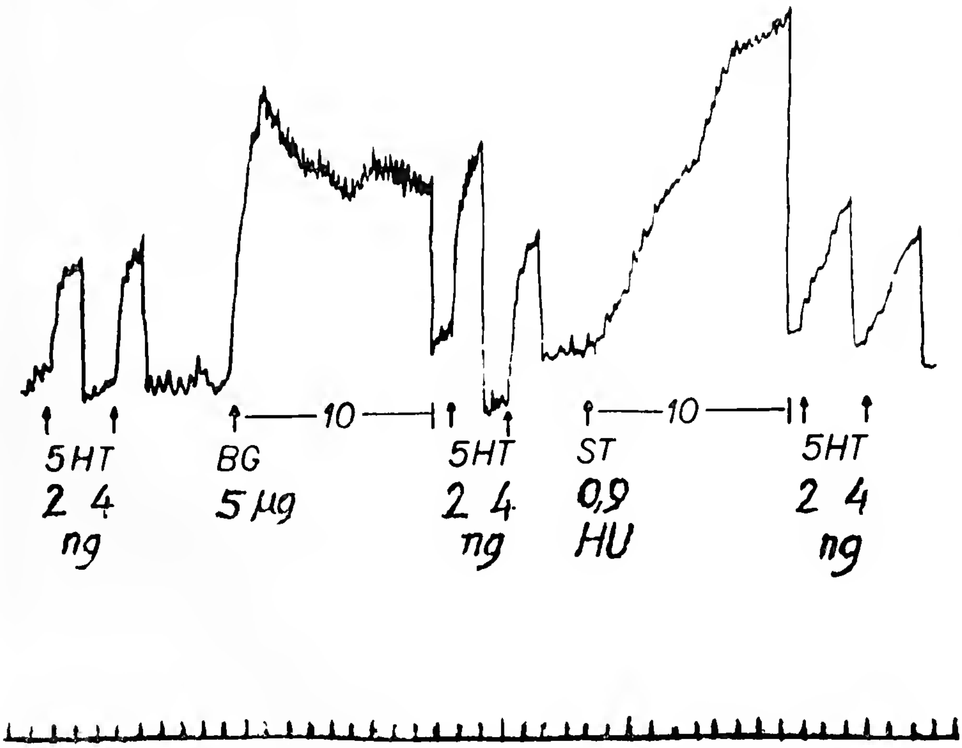

Fig. 3 — Effect of snake venom from *Bitis gabonica* (BG) on the contraction elicited by Staphylococcal alpha toxin (ST) administration.

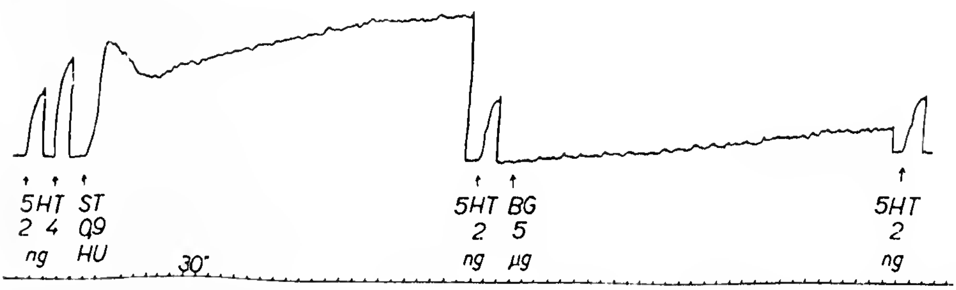

Fig. 4 — Effect of Staphylococcal alpha toxin on the contraction elicited by snake venom from *Bitis gabonica* (BG) on the isolated rat stomach.

When *Clostridium perfringens* toxin was administered to the stomach strip a slow contraction develops after latency period. A second administration of this toxin was much less effective and the organ lost also its sensitivity to originally active doses serotonin (Fig. 5). Also it was impossible to elicit a contraction with Staphylococcal alpha toxin (Fig. 6). The application of Staphylococcal alpha toxin prior to *Clostridium perfringens* toxin administration caused insensitivity of tissue to *Clostridium perfringens* toxin (Fig. 7).

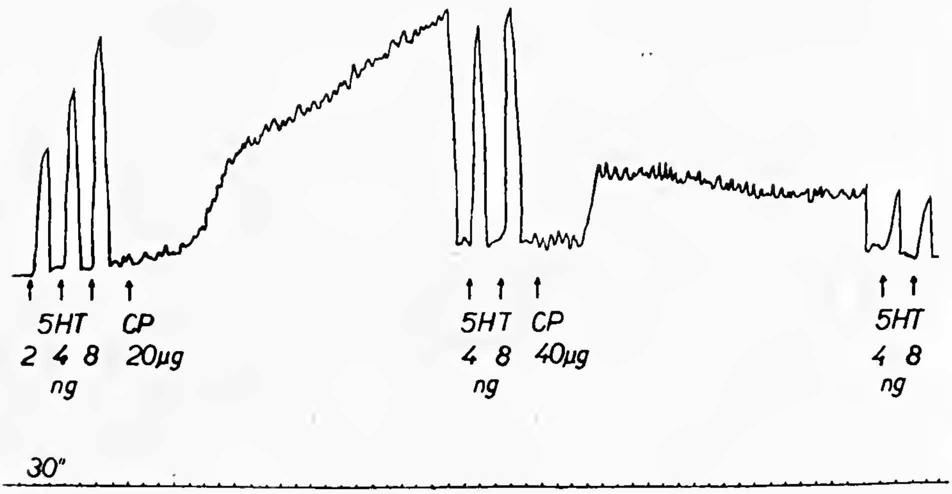

Fig. 5 — Effect of *Clostridium perfringens* (CP) toxin on the isolated rat stomach preparation.

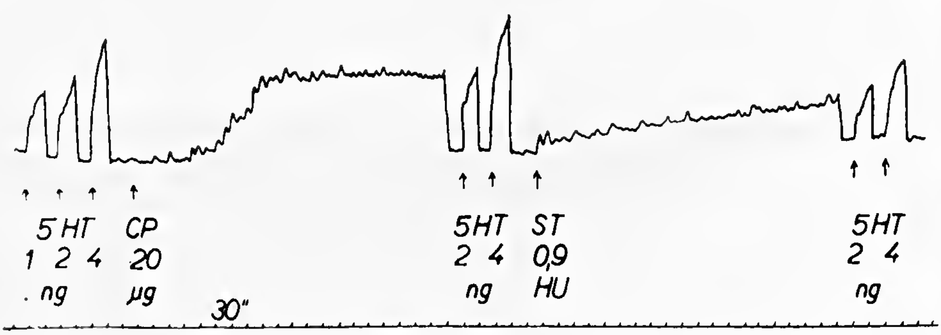

Fig. 6 — Effect of *Clostridium perfringens* toxin (CP) on the contraction elicited by Staphylococcal toxin (ST) on the isolated rat stomach.

Discussion

From the results described above it would seem that there are two clearly different phases of the contraction and it might be that they are due to two different components in the Staphylococcal culture filtrates which contain phospholipases (3, 4). It is not yet clear at present to which toxin (alpha or beta) they belong. We therefore used a snake venom rich in phospholipase A and *Clostridium perfringens* toxin rich in phospholipase C to try to contribute at least

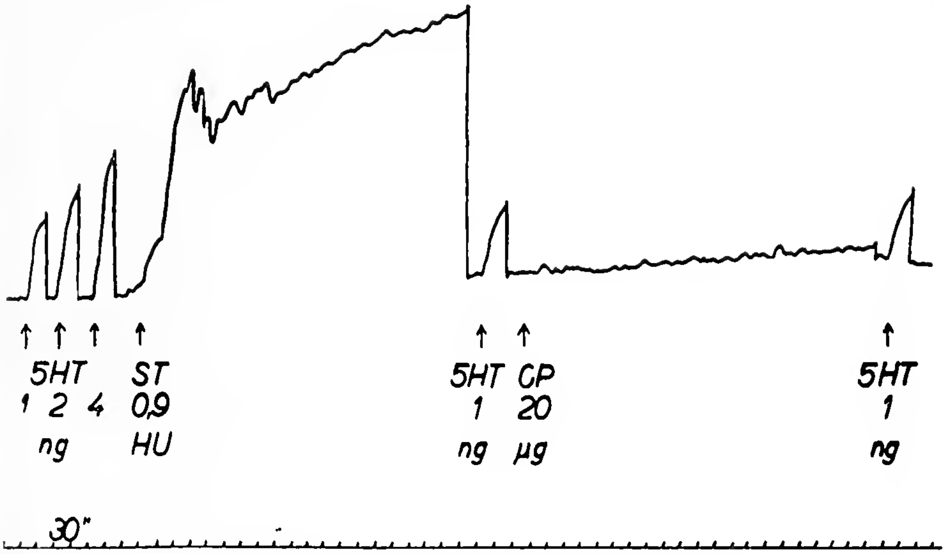

Fig. 7 — Effect of Staphylococcal toxin (ST) on the contraction elicited on the rat stomach preparation by *Clostridium perfringens* toxin.

indirectly to the question whether the observed spasmogenic activity would be attributable to phospholipases activity.

Venom toxin from *Bitis gabonica* does not prevent Staphylococcal alpha toxin from its activity but the character of contraction is different. The first rapid phase is not so evident. On the other hand Staphylococcal alpha toxin does prevent the snake venom from its activity completely. This would mean that part of Staphylococcal alpha toxin action corresponding to the rapid phase on isolated rat stomach are similar to action of venom toxin from *Bitis gabonica* and could be attribute to phospholipase A action. Since *Clostridium perfringens* prevents Staphylococcal alpha toxin from its activity as well the Staphylococcal alpha toxin prevents the action of *Clostridium perfringens* it would be reasonable to assume that both toxins might act on the same place of the phospholipide molecule. Further investigations are in progress.

We hope that this small contribution testifies that combined studies of bacterial and animal venoms might be of considerable interest.

Acknowledgments — The authors are most grateful to Dr. A. W. Bernheimer for the gift of Staphylococcal alpha toxin and to Dr. F. Kornalik for the gift of venom from *Bitis gabonica*.

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49. NEPHROTOXIC ACTION OF SNAKE VENOMS

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Experimental investigations have shown that animal venoms exert a nephrotoxic effect (1, 2). Thus, the clinical findings in subacute snake poisoning have been confirmed (3, 4, 5, 6).

The effects of snake venoms on the kidney reveal a most complex pattern. Several toxic components of the venom act either directly or indirectly on renal cells. Toxic degeneration of glomerular cells produces nephritis (7, 1, 8), toxic alteration of tubular cells evokes nephrosis (9, 10, 11, 1, 12, 13, 14, 15). Toxic lysis and degeneration of red blood cells or muscle cells (malayan sea snakes) provoke hemoglobinuric (4, 16) or myoglobinuric (14, 17) nephrosis. Toxic alteration of nerve cells cause an increased susceptibility of kidneys to all kinds of damages. Thus, an aggravation of the above-mentioned nephrotoxic effects of snake venom occurs (18, 19). In addition, enzymatic cellular lysis and thrombosis provoke renal infarct (7, 3, 6). Furthermore, the elicitation of anaphylactoid shock which is regularly found following snake poisoning (20, 21, 22, 23) causes renal hypoxia by collaps and vasoconstriction (see Table I).

TABLE I — EFFECTS OF SNAKE VENOMS ON THE KIDNEY

A. TOXIC EFFECTS

Renal glomerular cells ⟶ Nephritis

Renal tubular cells ⟶ Nephrosis

Red blood cells ⟶ Hemoglobinuric nephrosis

Muscle cells ⟶ Myoglobinuric nephrosis

Nerve cells ⟶ Increased renal susceptibility

B. ENZYMATIC EFFECTS

Cellular lysis, Thrombosis } ⟶ renal infarct

Formation of anaphylatoxin, Mast cell degranulation } ⟶ anaphylactoid shock } ⟶ hypoxic nephrosis

Following administration of snake venoms, three types of renal damage can occur: glomerular alterations (nephritis), tubular alterations (nephrosis), and renal infarct. The glomerular alterations consist in inflammatory and degenerative changes. The tubular alterations — which seem to be the most important in toxinologic respect — are produced by a direct toxic action ("intermediate nephron nephrosis"), by extrarenal cell degeneration (hemoglobinuric nephrosis), and by vascular hypoxia (Table II).

TABLE II — RENAL LESIONS FOLLOWING ADMINISTRATION OF SNAKE VENOM

1. GLOMERULAR ALTERATIONS (NEPHRITIS)
   a. Degenerative changes
   b. Inflammatory changes
2. TUBULAR ALTERATIONS (NEPHROSIS)
   a. direct toxic effects (intermediate nephron nephrosis)
   b. secondary effects by extrarenal cell degeneration (hemoglobinuric, myoglobinuric nephrosis)
   c. vascular hypoxia
3. RENAL INFARCT

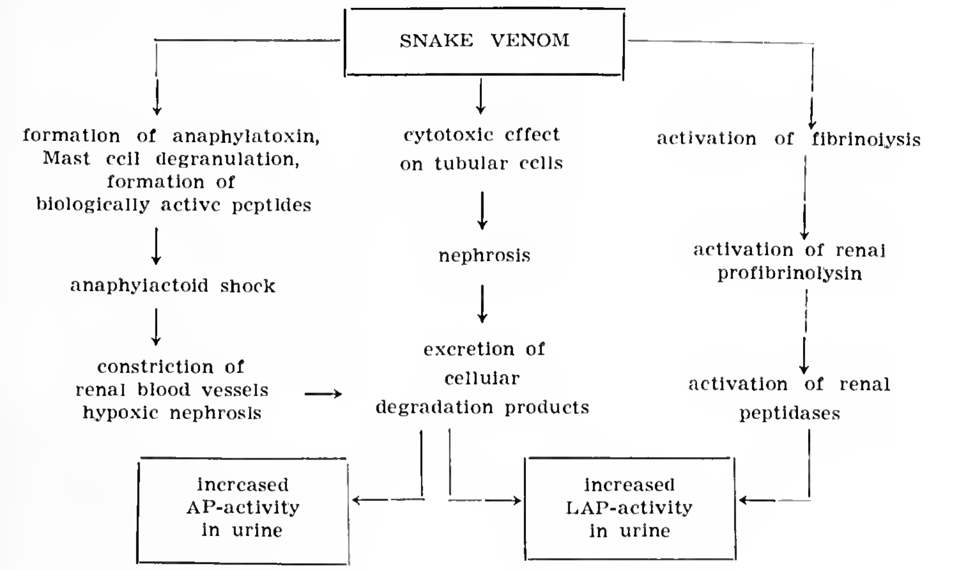
Until now, only pathologic and histologic examinations of kidneys in snake poisoning have been performed. In order to obtain information on the degree of renal damage *in vivo* and on the duration of nephrotoxic effect, biochemical investigations (24, 25, 26, 27, 15, 28) of renal function have been carried out following administration of snake venom.

After determination of normal alkaline phosphatase (AP) and "leucine aminopeptidase" (LAP) activities in 24-hours urine specimens (200 rats), 50 rats received a sublethal dosis of venom of *Agkistrodon piscivorus* LACEPEDE in saline (10 mg/kg subcutaneously). Daily determinations of enzymatic activities in urine were carried out for four days following administration of the snake venom. The enzymatic activities were calculated on 24-hours urine excretion.

As shown in Fig. 1, a significant increase in urinary AP- and LAP-activities occurs following injection of snake venom. On the second day, only a slight increase in urinary enzymatic activities is found. Beginning from the third day, normal activities were observed.

In 24-hours urine specimens following administration of snake venom, LAP-activity increases to the fivefold, AP-activity only to the twofold. AP-excretion depends upon renal tubular cells: following damage of these cells, an increase in urinary AP-activity occurs. The same statement applies for urinary LAP-activity: damage of tubular cells increases LAP-activity in urine. In addition, urinary LAP-activity is influenced by renal plasmine (see Table III). The activation of renal plasminogen provokes an activation of renal peptidases; this increases LAP-activity in urine.

TABLE III — MECHANISM OF INCREASE IN LAP- AND AP-ACTIVITY IN RAT URINE FOLLOWING ADMINISTRATION OF SNAKE VENOMS



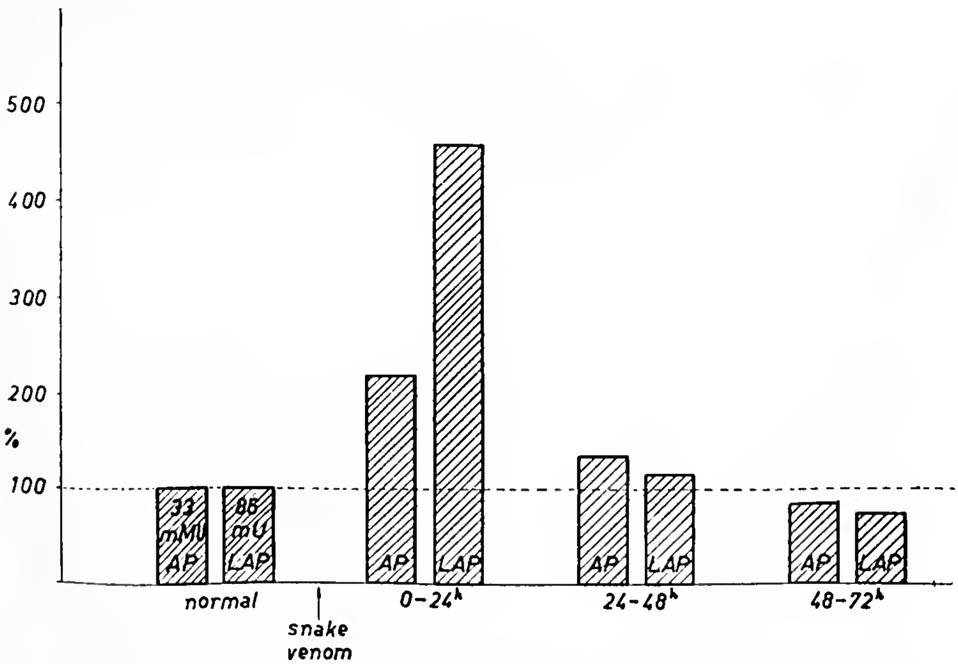


Fig. 1 — LAP- and AP-activities in rat urine (24 hours values) following injection of snake venom (*Agkistrodon piscivorus*). (Substrates: L-Leucyl-p-nitroanilide; Na-p-nitrophenylphosphate).

In anaphylactoid shock, an activation of fibrinolytic system occurs (23). In addition, certain snake venoms produce a direct activation of fibrinolytic system (29). Tubular damage by snake venom increases AP- and LAP-activities in urine. Activation of renal plasminogen causes a further increase in LAP-activity in urine.

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